DIFFERENTIAL INHIBITION OF RESTRICTION ENZYMES BY DIETARY MUTAGENS

(Generation of oxygen free radicals and degradation of DNA by methylglyoxal and quercetin)

A disseration submitted in partial fulfilment of the requirements for the degree of

MASTER OF PHILOSOPHY

IN

BIOCHEMISTRY

FABEHA FAZAL

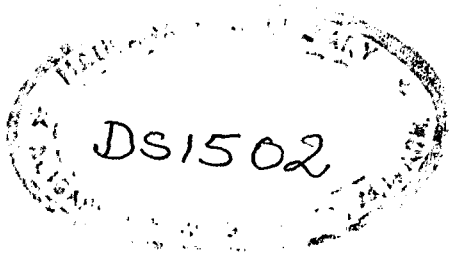
DEPARTMENT OF BIOCHEMISTRY

FACULTY OF LIFE SCIENCES

ALIGARH MUSLIM UNIVERSITY

ALIGARH

1989



Department of Biochemistry
Faculty of Life Sciences
ALIGARH MUSLIM UNIVERSITY,
ALIGARH-202002
U. P. INDIA

Date 4:11:89

CERTIFICATE

I certify that the work presented in this dissertation has been carried out by Miss. Fabeha Fazal under my supervision and is suitable for the award of M.Phil. degree in Biochemistry.

S Hadi

(S.M. Hadi)
Professor of Biochemistry

With love
to
my parents

ACKNOWLEDGEMENTS

I wish to express my heartiest gratitude to Prof. S. M. Hadi for his unending support, excellent guidance and encouragement throughout the course of this study. I am especially grateful to him for many stimulating ideas and for his helpful suggestions in the interpretation of the results.

I am highly thankful to Prof. A.M. Siddiqui and Prof.M. Saleemuddin, Chairman, Department of Biochemistry, for their persistent interest and generous availability of research facilities.

I am grateful to Drs. Masood Ahmad, A.N.K.Yusufi, (Mrs.) B. Bano, Fasih Ahmed, Saad Tayyab, Qayyum Hussain, Javed Musarrat and (Miss.) Imrana Naseem for their valuable suggestions at all levels.

My special and heartful thanks to Dr. Arshad Rahman for his immense help, encouragement and infinite co-operation throughout.

My gratuituous thanks to Drs. Shahabuddin Khalil, Maqbool Ahmed, Tariq Khan, Miss. Rana Zaidi, Tauseef Zehra, Tabassum Khan, Afshan Naheed, Shabana Iqrar, Zehra Rehana, Asma Zaidi, Rekha Das, Shabana Islam, Mana Mirza, Messers Shafat Qadri, Khalid Majid Fazili, Mohammed Saeed, Shahid Hussain, Rashid Butt and Abdul Mallick for their friendly co-operation always.

Financial assistance from Council of Scientific and Industrial Research is thankfully acknowledged.

Last but not the least to my dear parents and my brothers, Faizy, Adeeb, Faisal and Farrukh for their patience and stimulating interest in the persual of my research endeavour.

Fabeha Fazal

(Fabeha Fazal)

CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

INTRODUCTION

It is generally accepted that a high proportion of human cancers is attributable to environmental agents, mainly environmental chemicals. The distribution of potential carcinogens in the environment is essentially ubiquitous. The human diet contains a variety of naturally occurring mutagens and carcinogens (Ames, 1983). The predominance of certain foods in some countries has been related to the incidence of certain types of cancers in their populations. Therefore dietary mutagens have attracted considerable interest in the last decade and a number of studies on dietary practices in relation to cancer have been undertaken. These studies suggest that a greater intake of fibre rich cereals, vegetables, fruits and a lower consumption of fat rich products and alcohol would be advisable (Doll and Peto,1981; Peto and Schneiderman, 1981). Although quite a large number of dietary components have been evaluated in microbial and animal test systems,there is still a lack of definitive evidence about their carcinogenicity and mechanism of action. A majority of chemical carcinogens are known to form covalent adducts with DNA and there is a large body of evidence implicating DNA as a critical target in chemically induced cancer (Miller, 1978; O'Connor, 1981). In order to understand carcinogenesis at the molecular level,it is essential to determine the conformational changes in the target macromolecules and relate these findings to possible aberrations in the functioning of modified macromolecules. Of late,there has also been an increasing interest in oxygen radicals and lipid peroxidation as a source of damage to DNA and therefore as promoters of cancer (Harman,1981; Gensler and Bernstein, 1981; Totter,

1980; Tappel, 1980). In addition, mammalian systems have evolved many defence mechanisms as protection against mutagens and carcinogens. The most important of such mechanisms may be those against oxygen radicals and lipid peroxidation.

Mutagens and carcinogens in dietary plant material:

It is obvious that food is a very complex substance to which humans are exposed. Most people perceive food substances of natural origin as free of risk. Such acceptance is largely based on faith because our objective knowledge on this topic is relatively poor.A large number of chemicals are synthesized by plants, presumably as a defence against a variety of invasive organisms, such as bacteria, fungi and insects (Kapadia,1982; Clark, 1982; Pamukcu et al.,1980; Stich et al., 1981a). The number of these toxic chemicals is extremely large and new plant chemicals are being continuously discovered (Jadhav et al., 1981; Griesebach and Ebel, 1978). It has been known for many years that plants contain carcinogens and a number of edible plants have shown experimental carcinogenic activity for several species and various tissues.Wide use of recently discovered short term tests for detecting mutagens (Ames,1979; Stich and San,1981) and a number of animal cancer tests on plant substances have contributed to the identification of many natural mutagens and carcinogens in the human diet (Kapadia, 1982). Some examples of most frequently ingested compounds are discussed below.

Safrole and estragole are related compounds, which occur in certain spices and essential oils and are weak hepatocarcinogens (Fen-

aroli, 1971; Guenther and Althausen, 1949). Recent studies have implicated 1'-hydroxysafrole and 1'-hydroxyestragole, respectively as proximate carcinogenic metabolites of safrole and estragole (Drinkwater et al.,1976; Borchert et al., 1973). Eugenol and anethole are structurally related to safrole and estragole and are widely used as flavouring agents or as food additives. Black pepper contains small amounts of safrole and large amounts of a closely related compound piperine (Concon et al., 1979). Extracts of black pepper cause tumours in mice at a number of sites at a dose equivalent to 4 mg of dried pepper per day given for 3 months.

Ivie et al. (1981) have reported that linear furocoumarins (psoralens),which are widespread in plants of the Umbelliferae family, are potent light activated carcinogens and mutagens. Three of the most common phototoxic furocoumarins are psoralen, xanthotoxin and bergapten. In addition to Umbelliferae, psoralen also occurs in plants from several other families (Ivie, 1978). Psoralens are potent photosensitizers and highly mutagenic in the presence of activating long wavelength UV light. They readily intercalate into duplex DNA where they form light induced mono- or diadducts with pyrimidine bases.Psoralen, in the presence of light, is also effective in producing oxygen radicals (Ya et al., 1982).

Pyrrolizidine alkaloids are naturally occurring carcinogens and have been found in some fifty species of the families Compositae, Boraginaceae and Leguminosae (Schoental,1982), which are used as foods or herbal remedies. Several of these alkaloids are hepatotoxic and

certain hepatotoxic pyrrolizidine alkaloids are also carcinogenic (Hirono et al., 1977; Shoental, 1976). Testing of pure pyrrolizidine alkaloids for carcinogenicity has been extensive for reasons of a limited supply of these chemicals. However,a number of these alkaloids have been reported to be mutagenic (Clark, 1960) in Drosophila and Aspergillus system (Alderson and Clark, 1966). Recently, Mori et al. (1985) have used a hepatocyte primary culture-DNA repair test to screen seventeen pyrrolizidine alkaloids for their DNA damaging property. This test is highly responsive to carcinogenic pyrrolizidine alkaloids (Williams et al., 1980). Among the results obtained by these authors is the indication of a species difference in liver bioactivation of these alkaloids. This implies that there may be species difference in the carcinogenic potential of pyrrolizidine alkaloids.

Edible mushrooms contain various hydrazine derivatives in relatively large amounts. Most hydrazines that have been tested have been found to be carcinogenic and mutagenic. The most common commercial mushroom, Agaricus bisporus contains about 300 mg of agaritine, the -glutamyl derivative of the mutagen 4-hydroxymethylphenylhydrazine, per 100 g of mushrooms as well as smaller amounts of the closely related carcinogen N-acetyl-4-hydroxymethylphenylhydrazine (Toth et al., 1982). Some agaritine is metabolized by the mushroom to a diazonium derivative, which is a potent carcinogen and is also present in the mushroom in smaller amounts. Many hydrazine carcinogens may act by producing oxygen radicals (Hochstein and Jain, 1981).

A number of 1,2-dicarbonyl compounds e.g., maltole, kojic acid,

ethylmaltole, diacetyl and glyoxal have been found to be mutagenic in the Salmonella/Microsome assay. Several compounds in this class are of toxicological interest because they occur in various foods. For example, maltole is a product of carbohydrate dehydration and is present in coffee, soyabeans and baked cereals such as bread. Kojic acid is a metabolite of many microorganisms including several fungi used in food production, while diacetyl is an aroma component of butter, beer, coffee, etc. (Fishbein, 1983).

A number of furans, such as 2-methylfuran, 2,5-dimethylfuran, furfural, 5-methylfurfural and 2-furylmethylketone are found in numerous food products including meat, milk products, various nuts, tea and coffee (Maga,1979). Stich et al. (1981b) have reported that these furans induced relatively high frequencies of chromatid breaks and chromatid exchanges when they were exposed to cultured Chinese hamster ovary (CHO) cells in the absence of a liver microsomal preparation. The clastogenic doses of many of the furans were relatively high (100-3900 ppm), whereas the concentration in food products was relatively low. However, Stich et al. (1981b) cautioned that the furans are not the only genotoxic chemicals in the complex mixture of heated, roasted or boiled food products and even if the furans do not pose a serious health hazard by themselves due to their small amounts in most food items, they do contribute significantly to the total genotoxicity of many consumable foods and beverages.

In addition to pyrrolizidine alkaloids, certain glycoalkaloids found in potato, such as solanine and chaconine, have been reported to

be highly toxic as they are strong inhibitors of cholinesterase (Jadhav et al.,1981). Pyrrolizidine alkaloids and other glycoalkaloids can reach levels which can be lethal to humans in potatoes that are diseased or exposed to light (Katsui et al.,1982).

Cyclopropenoid fatty acids,present in cotton seed and other oils, have been reported to be carcinogenic and mitogenic having various toxic effects in farm animals. Among these, sterculic acid and malvalic acid are widespread in the human diet. They are also potentiaters of carcinogenicity of aflatoxins (Hendricks et al., 1980). Human exposure to these fatty acids results from the consumption of products of animals fed on cotton seed. Another major toxin in cotton seed is gossypol, which accounts for about 1 % of its dry weight. Gossypol causes male sterility through formation of abnormal sperm and is carcinogenic as well (Xue, 1980). It is a potent initiator and also promoter of carcinogenesis in mouse skin (Haroz and Thomassan, 1980). Gossypol has been tested in China as a possible male contraceptive as it is inexpensive and causes sterility during use. Its mode of action as a spermicide is presumably through the production of oxygen radicals.

A number of quinones and their phenolic precursors are found in the human diet and have been shown to be mutagens (Stich et al.,1981b; Brown, 1980; Levin et al., 1982). Quinones are quite toxic as they can act as electrophiles or accept a single electron to yield the semiquinone radicals which can react directly with DNA or generate superoxide radicals (Morimoto et al., 1983; Kappus and Sies, 1981).

Many dietary phenols can autoxidize to quinones generating hydrogen peroxide at the same time. The amounts of these phenols in human diet are appreciable, for example, catechol which is mainly derived from metabolism of plant substances and is a potent promoter of carcinogenesis and an inducer of DNA damage (Carmella et al.,1982).

In addition, there are many other dietary compounds which have been shown to be mutagenic and carcinogenic in various test systems. Allylisothiocyanate, a major flavour ingredient of mustard oil, is one of the main toxins of mustard seeds and has been shown to be a carcinogen in rats (Dunnick et al., 1982). Phorbol esters, present in plants of Euphorbiacea family, are potent promoters of carcinogenesis and cause nasopharyngeal and oesophageal cancers (Hecker, 1981). A variety of carcinogens and mutagens are present in mold contaminated food grains, nuts and fruits. Some of these,such as various aflatoxins, are among the most potent carcinogens and mutagens known (Hirono, 1981; Tazima, 1982). Nitrosoamines and other nitroso compounds formed from nitrate and nitrites in food have been directly related to the incidence of stomach and oesophageal cancer. Nitrates are present in large amounts in spinach, radish, lettuce and beans (Magee, 1982). Although alcohol is not a constituent of a normal human diet, in view of its widespread use, it would be relevant to mention its toxic role. Alcohol has long been associated with the cancer of mouth, pharynx and liver (Tuyns et al., 1982). Alcohol metabolism generates acetaldehyde, which is a mutagen and possibly a carcinogen (Stich and Rosin, 1983; Campbell and Fantel, 1983). It also generates radicals that produce

lipid hydroperoxides and other mutagens and carcinogens (Winston and Cederbaum, 1982; Videla et al., 1982).

Dietary fat - a possible source of carcinogens:

Fat accounts for approximately 40 % of the calories in the human diet. There is epidemiological evidence relating high fat intake with colon and breast cancer. Animal studies have indicated that high dietary fat is a promoter and a presumptive carcinogen (Kinlen, 1983; Fink and Kritchevsky,1981; Welsch and Aylsworth,1983).Two plausible mechanisms, involving oxidative processes, have been considered to account for the relationship between high fat intake and the occurrence of cancer and heart diseases. According to the first mechanism, rancidity of fat yields a variety of mutagens and carcinogens,such as fatty acid hydroperoxides,cholesterol hydroperoxides,fatty acid epoxides and aldehydes (Simic and Karel, 1980; Bischoff, 1969; Petrakis et al., 1981; Imai et al.,1980; Ferrali et al.,1980). Alkoxy and hydroperoxy radicals are also formed (Pryor,1976-1982). Therefore the colon and digestive tract are exposed to a variety of fat derived carcinogens. The second possible mechanism involves hydrogen peroxide, which is generated by the oxidation of dietary fatty acids by peroxisomes. Each oxidative removal of two carbon unit generates one molecule of hydrogen peroxide,a known mutagen and carcinogen (Reddy et al., 1982; Plain, 1955). Some hydrogen peroxide may escape the catalase in the peroxisomes and thus contribute to the supply of oxygen radicals (Speit et al., 1982; Jones et al., 1981). Oxygen radicals in turn can damage DNA and can start the rancidity chain reaction, which leads to the production of

the mutagens and carcinogens mentioned above (Pryor, 1976-1982).

Mutagens and carcinogens produced in cooking:

Sugimura and his colleagues (1978, 1979) as well as others (Pariza et al.,1983) have reported that the burnt and browned materials from heating protein during cooking is highly mutagenic. Pyrolysis of protein produces strong frameshift mutagens that require metabolic activation by rat liver S9 fraction (Nagao et al., 1977). Pyrolysates of amino acids also show various mutagenic activities (Matsumoto et al., 1977). Among the various amino acids, the pyrolysate of tryptophan has been found to be most mutagenic followed by those of serine, glutamic acid, ornithine and lysine.

Pyrolysates of various sugars, such as glucose, arabinose, fructose and sorbitol, are all mutagenic in S. typhimurium system without metabolic activation. Pyrolysate of glucose was found to contain acetaldehyde and glyoxal which are mutagenic to S. typhimurium (Nagao et al., 1978). Caramel, which is sugar derived and widely used as a food colouring and flavouring agent is also mutagenic in Salmonella test systems but had no carcinogenic effect when fed to rats as 6 % of the diet for two years (Evans et al., 1977). Coffee contains a considerable amount of burnt material including the mutagenic pyrolysis product methylglyoxal (Sugimura and Sato, 1983). One cup of coffee also contains about 250 mg of the natural mutagen chlorogenic acid (Stich et al., 1981a) and about 100 mg of caffeine which can cause birth defects at high levels in several experimental species (Fabro, 1982). There is inconclusive evidence to suggest that heavy

coffee drinking is associated with cancer of the ovary, bladder, pancreas and the large bowel (Trichopoulos et al., 1981). Rancidity reaction of cooking oils and animal fat is accelerated during cooking, thus increasing intake of mutagens and carcinogens (Simic and Karel, 1980).

Food additives:

Sodium nitrite is extensively used as a preservative in meat, fish and cheese. A possible formation of nitrosamines from amines,present in or derived from the diet, occurs by reaction with nitrous acid at acidic pH.In humans, gastric juice attains a pH of nearly 1.0. Such high concentration of hydrogen ions gives rise to the nitrosyl cation No^{+}, which is a highly reactive nitrosylating agent. Nitrous acid itself is a known mutagen for various bacterial and fungal cells. Its mutagenicity is presumably related to the deamination of adenine and cytosine (Fishbein et al.,1970). Sodium bisulphite is used as a bacterial inhibitor in a variety of beverages and as a preservative in canned fruits and vegetables. The bisulphite anion reacts, rather specifically, with uracil and cytosine, within single-stranded regions of DNA and RNA. It is also mutagenic to bacteria and bacteriophages (Singer, 1983). EDTA and its alkali salts are widely used as sequestrants in various foods. They are useful as antioxidants due to their property of forming poorly dissociable chelate complexes with trace quantity of metal ions such as copper and iron in fats and oils. EDTA has been shown to induce chromosome aberrations and breakage in various plant species.

Saccharin was synthesized in the last century and since then it

has been widely used as an artificial sweetener. Reports on the mutagenicity and carcinogenicity of saccharin are conflicting and there is some suggestion that these activities are thought to be due to impurities present in saccharin preparations (Kramers,1975). The possibility of an in vivo conversion of saccharin into a mutagenic metabolite has also been suggested (Batzinger et al., 1977). Another artificial sweetener, which was widely used but is now banned in USA and many other countries,is cyclamate. Cyclamate induces chromosome breakage in cells of several plants and animal species. It is converted in vivo into cyclohexylamine, which is also an inducer of chromosome breaks (Fishbein et al., 1970).

Oxygen radicals and cancer:

One of the theories of etiology of cancer which is being widely accepted,holds that the major cause is damage to DNA by oxygen radicals and lipid peroxidation (Ames, 1983; Totter, 1980). Several enzymes produce superoxide anion ($O_2^{\cdot -}$) during the oxidation of their substrates, for example, xanthine oxidase and peroxidase (Buettner et al., 1978; Duran et al.,1977). Numerous substances such as reduced flavins and ascorbic acid upon autoxidation produce superoxide anion. This radical further accepts an electron from a reducing agent, such as thiols to yield peroxide (H_2O_2). There is in vitro evidence that H_2O_2 may then react with certain chelates of copper and iron to yield the highly reactive hydroxyl free radical ($OH^{\cdot}$) (Wolff et al., 1986). That the superoxide anion actually appears in metabolism is confirmed by the ubiquitous occurrence of superoxide dismutase. Indeed, certain white blood cells

generate superoxide deliberately by means of a specialized membrane bound NADPH oxidase and this participates in the killing of microorganisms and tumour cells (Wolff et al., 1986).

It has been suggested that certain promoters of carcinogenesis act by generation of oxygen radicals, this being a common property of these substances. Fat and hydrogen peroxide are among the most potent promoters (Welsch and Aylsworth, 1983). Other well known cancer promoters are lead, calcium, phorbol esters, asbestos and various quinones. Inflammatory reactions lead to the producton of oxygen radicals by phagocytes and this is the basis of promotion by asbestos (Hatch et al., 1980). Many carcinogens which do not require the action of promoters and are by themselves able to induce carcinogenesis (complete carcinogens), also produce oxygen radicals (Demopoulos et al., 1980). These include nitroso compounds, hydrazines, quinones and polycyclic hydrocarbons. Much of the toxic effect of ionizing radiaton damage to DNA is also due to the formation of oxygen radicals (Totter,1980). The mechanism of action of promoters involves the expression of recessive genes and an increase in gene copy number through chromosome breaks and creation of hemizygosity (Kinsella, 1982; Varshavsky, 1981).Promoters also cause modification of prostaglandins which are intimately involved in cell division, differentiation and tumour growth (Fischer et al.,1982). Most data on radical damage to biological macromolecules concern with the effects of radiation on nucleic acids because of the possible genetic effects. However, in view of the catalytic role of enzymes, damage to proteins is also considered important. It has been

suggested that primary oxygen radicals, produced in cells and their secondary lipid radical intermediates, modify and fragment proteins. The products are often more susceptible to enzymatic hydrolysis leading to accelerated proteolysis inside and outside the cells (Wolff et al., 1986).

Anticarcinogens:

The protective defence mechanisms against mutagens and carcinogens include the shedding of surface layer of the skin, cornea and the alimentary canal. If oxygen radicals play a major role in DNA damage, defence against these agents is obviously of great importance (Totter, 1980). The major source of endogenous oxygen radicals are hydrogen peroxide and superoxide which are generated as side products of metabolism (Pryor, 1976-1982). In addition,oxygen radicals also arise from phagocytosis after viral and bacterial infection or an inflammatory reaction (Tauber, 1982). The exogenous oxygen radical load is contributed by a variety of environmental agents (Pryor, 1976 - 1982). The enzymes that protect cells from oxidative damage are superoxide dismutase, glutathione peroxidase (Pryor, 1976- 1982) D.T. diaphorase (Lind et al.,1982) and glutathione transferases (Warholm et al., 1981). In addition to these enzymes, some small molecules in the human diet act as antioxidative agents and presumably have an anticarcinogenic effect. Some of these compounds are discussed below.

Tocopherol (vitamin E) is an important trap of oxygen radicals in membranes (Pryor, 1976 - 1982) and has been shown to decrease the carcinogenic effect of quinones, adriamycin and daunomycin which are

toxic because of free radical generation (Ames, 1983). Protective effect of tocopherols against radiation induced DNA damage and dimethylhydrazine induced carcinogenesis have also been observed (Beckman, et al., 1982). β-carotene is a potent antioxidant present in the diet and is important in protecting lipid membranes against oxidation. Singlet oxygen is a highly reactive form of oxygen, which is mutagenic and is mainly generated by pigment mediated transfer of energy of light to oxygen. Carotenoids are free radical traps and are remarkably efficient as quenchers of singlet oxygen (Packer et al., 1981). β-carotene and similar polyprenes are also the main defence in plants against singlet oxygen generated as a byproduct of the interaction of light and chlorophyll (Krinsky and Deneke, 1982). Carotenoids have been shown to be anticarcinogens in rats and mice and may also have a similar effect in humans (Mathews-Roth,1982; Peto et al.,1981). Glutathione is present in food and is one of the major antioxidants and is antimutagenic in cells. Glutathione transferases are a major defence against oxidative and alkylating carcinogens (Warholm et al., 1981). Dietary glutathione is an effective anticarcinogen against aflatoxins (Novi ,1981). The cellular concentration of glutathione is influenced by dietary sulphur amino acids (Tateishi et al., 1981). Selenium, which is present in the active site of glutathione peroxidase, is another important dietary anticarcinogen. Glutathione peroxidase is essential for destroying lipid hydroperoxides and endogenous hydrogen peroxide and therefore helps to prevent oxygen radical induced lipid peroxidation (Flohe, 1982). Several heavy metal toxins,

such as Cd^{2+} (a known carcinogen) and Hg^{2+} decrease glutathione peroxidase activity by interacting with selenium (Flohe, 1982). Some other dietary antioxidants include ascorbic acid and uric acid. The former has been shown to be anticarcinogenic in rodents treated with UV light and benzo(a)pyrene (Hartman, 1982). Uric acid is present in high concentrations in the blood of humans and is a strong antioxidant (Ames et al.,1981). A low uric acid level has been considered a risk factor in cigarette caused lung cancer; however, too high levels may cause gout.

In addition, edible plants contain a variety of substances such as phenols that have been reported to inhibit or enhance carcinogenesis and mutagenesis in experimental animals (Ames, 1983). The inhibitory action of such compounds may be due to the induction of cytochrome P-450 and other metabolic enzymes (Boyd et al., 1982). The optimum levels of dietary antioxidants have not been determined; however,there might be considerable variation among individuals. On the other hand, high doses of such compounds may lead to deleterious side effects. The differences in cancer rates of various populations are generally considered to be due to environmental and life style factors such as smoking, dietary carcinogens and promoters. However, these differences may also be due, in good part, to insufficient amounts of anticarcinogens and other protective factors in the diet (Maugh, 1979).

In the past two decades, there has been much emphasis on the induction of cancer by occupational and industrial pollution factors. There is growing recognition, however, that these may account for only

a small fraction of human cancers. It is becoming increasingly clear from epidemiological and laboratory data that diet is an important factor in the etiology of certain human cancers. It has been suggested by Doll and Peto (1981) that in the United States diet accounts for 35 % of cancer deaths. According to these authors, there are five possible ways whereby diet may effect the incidence of cancer;(i) ingestion of powerful direct acting carcinogens or their precursors (ii) affecting the formation of carcinogens in the body; (iii) affecting transport, activation or deactivation of carcinogens; (iv) affecting "promotion" of cells that are already initiated and (v) overnutrition. Normal individual consumption of potentially mutagenic substances per day from foods and beverages is estimated to be between 1 to 2 gm. In addition,the endogenous conditions favour the formation of still more mutagens in vivo in humans (Oshshima and Bartsch, 1981).

SCOPE OF THE WORK PRESENTED

Mutagenicity of methylglyoxal:

Methylglyoxal (MG), also known as pyruvaldehyde or acetylformaldehyde, is a ketoaldehyde and may arise in the cell both enzymatically (Cooper and Anderson,1970; Elliot,1960; Sato et al.,1980) and nonenzymatically (Riddle and Lorenz, 1968) from free trioses. Besides, it has also been reported to be present in various foods, such as roasted coffee,beans, tea, whisky and soy sauce (Sugimura and Sato, 1983). Whether the enzymatic MG formation actually occurs in mammals has been controversial for many years (Meyer, 1953; Bonsignore et al., 1976; Salem, 1975; Van Eys et al.,1962; Riddle and Lorenz, 1968).The isolation of MG synthase from the enterobacteriaceae (Cooper,1974; Yuan and Gracy,1977) and its presence in rat liver cells (Sato et al., 1980) confirmed that MG can be formed enzymatically from triose phosphates. Riddle and Lorenz (1968) observed that MG formation from both dihydroxyacetone phosphate (DHAP) and DL-glyceraldehyde is accelerated by polyvalent cations at physiological pH values.

Szent Gyorgyi (1967, 1977) proposed that MG interacts with the highly reactive sulfhydryl groups that may participate in the regulation of cell division in tissues and that this MG-SH complex can arrest cell division in rapidly dividing cells. MG and other similar aldehyde compounds exert significant effects on certain cancers by reducing the ascites fluid formation, prolonging the survival time of animals bearing those tumours and decreasing the mitotic index of

normal and tumour cells (Jerzykowski et al., 1970; Fenselau and Long, 1976; Dianzani et al., 1978, 1980). The uncontrolled proliferation of tumour cells is supposed to be due to destruction of MG (Szent Gyorgyi, 1977) by two enzymes, namely glyoxalase and ∝-ketoaldehyde dehydrogenase, which catalyze its oxidation to lactate (Racker, 1951) and pyruvate (Monder,1967), respectively.

In millimolar amounts, MG exerts several damaging effects on various biochemical parameters (Dianzani, 1979) and has been found to inhibit in vitro the growth of a variety of mammalian cell lines (Gregg, 1968; Klamerth, 1968; Scaife, 1969). It has been shown to be mutagenic in Salmonella typhimurium TA100 (Kasai et al., 1982; Fujita et al., 1985) and to induce DNA repair in the pyloric mucosa of rats by gastric incubation (Furihata et al.,1985). Recently, in a screening performed to elucidate the DNA damaging activities of a series of biotic and xenobiotic aldehydes, Brambilla et al. (1984) found that exposure of cultured mammalian cells to non-toxic concentrations of MG resulted in the formation of macromolecular cross-links, mainly of the DNA protein type (Brambilla et al., 1985). Sister-chromatid exchanges in Chinese hamster ovary cells (Faggin et al., 1985) and mutagenicity in cultured Chinese hamster lung cells,assessed by using diptheria toxin resistance as a selective marker have also been reported (Nakasato et al., 1984). MG causes a dose-dependent increase in the frequency of HGPRT deficient mutants in V79 cells (Cajelli et al., 1987). Efforts have also been made to investigate the effect of MG on the microtubular system in order to understand the mechanisms of its antiprolifera-

tive activity (Gabriel et al., 1985). Studies in our laboratory have shown that the reaction of MG with DNA leads to the formation of strand breaks and interstrand crosslinks as a function of MG concentration and also its time of reaction with DNA (Rahman et al.,1989b).

Fugita et al. (1985) have recently reported that MG considerably enhances the mutagenicity of H_2O_2 which is otherwise weakly mutagenic in Salmonella typhimurium TA100. In view of the above observation it was of obvious interest to examine if MG generates oxygen free radicals in solution, which are responsible for its increased mutagenicity. Using an absorbance method, the present work demonstrates that MG undergoes photo-oxidation leading to the generation of superoxide dependent hydroxyl radical formation through the Haber-Weiss reaction.

Mutagenicity of quercetin:

Of late, there has been increasing interest in naturally occurring compounds, ingested as part of the normal diet, which are potentially mutagenic/carcinogenic (Ames, 1983).One such class of compounds is the flavonoids which occur in large amounts in a wide range of food plants including many fruits, vegetables, tea, skin of tubers and roots (Hermann, 1976). There are many flavonoids in plants and the mutagenicity of more than seventy naturally occurring flavonoids has been tested (Nagao et al., 1978). Of these, quercetin was the strongest mutagen, followed by kaempferol,rhamnetin, galangin, isorhamnetin and fisptin (Brown, 1980; Nagao et al., 1981). It has been estimated that

the average daily intake of flavonoids in the American diet is about 1 gm and thus there is clearly a potential hazard. All these compounds except quercetin required metabolic activation by rat liver enzymes when tested in microbial systems (Nagao et al., 1978).The mutagenicity of quercetin was further enhanced by rat liver enzymes. This suggests that quercetin may interact directly with cellular DNA. Besides the microbial system, flavonoids especially quercetin and kaempferol have also been tested for mutagenicity in higher systems, such as rat, hamsters and Drosophila. However, in these systems there have been conflicting reports on the mutagenicity and carcinogenicity of quercetin (Hirono et al., 1981; Pamukcu et al., 1980; Watson, 1982). Whereas studies of Ambrose et al. (1952) reported quercetin to be non-carcinogenic to rats fed 1% quercetin for 410 days, more recent definitive studies of Pamukcu et al.(1980) demonstrated that quercetin was carcinogenic for the intestinal and bladder epithelium of the rat when fed as a basic grain diet of 0.1 % quercetin (of purity " 99 %) for 58 weeks. Although the mechanism of carcinogenicity of quercetin is not known, it has shown significant effects on DNA synthesis, lactate production and cyclic adenosine 3',5'-monophosphate level in neoplastic cells (Podhajcer et al., 1980).

Quercetin in common with other flavonoids is a candidate substance for the development of antiviral agents (Vanden et al.,1986; Van Hoof et al., 1984) and is a promising compounds for the inhibition of tumor invasion (Bracke et al.,1987). The mechanism by which quercetin exhibits its antitumor activity is not understood. Since

it is a frame-shift mutation in S. typhimurium (Ames, 1972) it has been argued that it might be an intercalating agents (Bjeldanes, 1977). However there are no chemical data to support this view. The genotoxicity of quercetin correlates with the ability of the substance to cause DNA strand scission in the presence of Cu(II) and molecular oxygen (Rahman et al.,1989a). Previous studies have suggested that flavonoids function as scavengers of reactive species of oxygen such as singlet oxygen (Takahama et al.,1984), superoxide anion (Takahama et al., 1983), and H_2O_2 (Takahama, 1984). From the studies on the effect of metal ions, antioxidants and pH on the mutagenicity of quercetin in S.typhimurium. Hatcher and Bryan (1985) concluded that this reaction is antimutagenic. Contrary to the above observations the strand scission reaction of quercetin is associated with the formation of a ternary complex of DNA, quercetin and Cu(II) (Rahman et al., 1989b), followed by a transient reduction of Cu(II) to Cu(I) and the generation of active oxygen species (Rahman et al., 1989a). These conflicting reports led me to investigate if quercetin is capable of generating oxygen free radicals in solution.

Using fluorescence and absorbance techniques it is reported here that quercetin upon photo-oxidation or metal-catalysed oxidation leads to the generation of superoxide dependent $OH^{\cdot}$ production through the Haber-Weiss reaction (Haber and Weiss, 1934; Beauchamp and Fridovich, 1970).

EXPERIMENTAL

MATERIALS

Chemicals used were obtained from the sources given against their names.

Chemical	Source
Agarose	Koch-light Laboratory, England.
Bovine serum albumin (BSA)	Sigma Chemical Co., U.S.A.
B-mercaptoethanol	Merck, Germany.
Catalase	Sigma Chemical Co., U.S.A.
Cupric chloride	B.D.H., India.
Deoxyribonucleic acid (Calf thymus type I)	Sigma Chemical Co., U.S.A.
Dimethyl sulphoxide (DMSO)	Merck, India.
DraI	New England Biolabs., U.S.A.
Ethylenediaminetetra-acetic acid (EDTA)	B.D.H., India.
EcoRI	New England Biolabs., U.S.A.
Ferric chloride	B.D.H., India.
Glycerol	B.D.H., India.
Lambda phage DNA	New England Biolabs., U.S.A.
Methylglyoxal	Sigma Chemical Co., U.S.A.
N-ethyl-N-nitrosourea (EtNu)	Sigma Chemical Co., U.S.A.
N-methyl-N-nitrosourea (MeNu)	Sigma Chemical Co., U.S.A.
Nitroblue tetrazolium (NBT)	Sisco Research Labs., Bombay.
Potassium dihydrogen orthophosphate	B.D.H., India.
Potassium hydroxide	Merck, Germany.

Quercetin	Sigma Chemical Co., U.S.A.
SmaI	New England Biolabs., U.S.A.
Spermine	Sigma Chemical Co., U.S.A.
Spermidine	Sigma Chemical Co., U.S.A.
Supercoiled plasmid pSK 275 DNA	A generous gift from Dr. Saleem Khan, University of Pittsburg, U.S.A.
Superoxide dismutase (SOD) (Bovine erythrocyte)	Sigma Chemical Co., U.S.A.
Thiobarbituric acid (TBA)	Loba Chemical Co., India.
Tris-(hydroxymethyl)-amino methane	Fluka, Switzerland.

METHODS

Detection of superoxide anion ($O_2^{\cdot-}$):

Superoxide anion was detected by the reduction of nitroblue tetrazolium (NBT) essentially as described by Nakayama et al (1983). A typical assay contained, in a total volume of 3.0 ml, 50 mM potassium phosphate, pH 7.8, 33 mM NBT, 0.1 mM EDTA and 0.06% triton X-100. The reaction was started by the addition of MG or quercetin. Immediately after mixing, the absorbance at 560 nm was measured under various experimental conditions against a blank which did not contain MG or quercetin. To confirm the formation of $O_2^{\cdot-}$, superoxide dismutase (SOD) was introduced into the solution before adding MG or quercetin.

Detection of hydroxyl radicals ($OH^{\cdot}$):

Determination of $OH^{\cdot}$ was done by the following two methods.

I. Aromatic hydroxylation:

The assay is based on the ability of OH to hydroxylate (attack) aromatic rings at an almost diffusion controlled rates and the measurement of hydroxylated products by a simple colorimetric method using salicylate (2-hydroxy benzoate) as a detector molecule (Richmond et al., 1981).

The reaction mixture (2.0 ml) contained the following reagents at the indicated concentrations. 2 mM salicylate, 0.1 mM EDTA, 0.1 mM $FeCl_3$ and 150 mM KH_2PO_4-KOH buffer, pH 8.0. The reaction was initiated by adding appropriate amount of MG or quercetin. In case of quercetin,

Fe(III) was replaced by Cu(II) and the tubes were incubated at room temperature for 2 hr. Reaction was stopped by adding 80 ul of 11.6 M HCl and 0.5 gm NaCl followed by 4.0 ml of chilled diethylether. The contents were mixed by Vortexing for 1 min. Next, 3.0 ml of upper (ether) layer was pipetted off and evaporated to dryness in a boiling tube at 40 C. The tubes were cooled and the residue dissolved in 0.25 ml of cold distilled water to which the following reagents were added in the order stated (a) 0.125 ml of 10% w/v TCA dissolved in 0.5 M HCl (b) 0.25 ml of 10% w/v sodium tungstate in water (c) 0.25 ml of 0.5% w/v $NaNO_2$(freshly prepared). After standing for 5 min, 0.5 ml of 0.5 M KOH was added and the absorbance at 510 nm was read exactly after 1 min.

II. Degradation of DNA and development of fluorescent thiobarbituric acid (TBA) adduct:

This method is based on the fact that degradation of DNA by OH results in the release of TBA-reactive material which forms a fluorescent adduct with TBA (Quinlan and Gutteridge, 1987). The following reagents were added in clean tubes in the order indicated; 0.4 ml of Tris-HCl buffer (0.1 M, pH 7.5), 0.4 ml DNA (2 mg/ml in 0.01 M Tris-HCl buffer pH 7.5, 0.01 M NaCl), and quercetin at concentrations indicated in the appropriate figures and tables. The reaction was started by the addition of appropriate amounts of metal salts (see figures and tables) and the resulting mixtures were incubated overnight at 37 C. In experiments where DNA was degraded by 1,10 phenanthroline the procedure as described by Gutteridge (1984) was followed.

After incubation of sample, 1.0 ml TBA (1% w/v in 0.05 M NaOH) and 11.0 ml of 28% w/v TCA were added. The tube contents were heated at 100 C for 15 min to develop the colour. The resulting TBAchromogen was extracted into 3.0 ml n butan-1-ol and the clear organic layer used for spectrofluorometric measurements. Fluorescence in the butanol extracts was measured at 553 nm following excitation at 532 nm using a Shimadzu spectrofluorometer.

Treatment of lambda DNA with quercetin and restriction enzyme digestion:

0.5 ug lambda phage DNA was incubated in a volume of 20 ul in Tris-HCl (10 mM, pH 8.0) with quercetin which was present at the molarities indicated in the text. The reaction mixtures were incubated at 37 C for 1 hr and then dialysed on 0.025 micron pore size millipore filter. The dialysed samples were digested with appropriate units of various restriction enzymes. The reaction was stopped by adding 10 ul sample buffer containing 0.2% SDS, 20% sucrose and 0.1% bromophenol blue. The resulting solution was electrophoresed on a 1.0% agarose gel containing 1 ug/ml ethidium bromide.

Preparation of crosslinked DNA

Crosslinked DNA was prepared as described by Notani (1975). To 10 ml DNA (2 mg/ml) in TNE (10 mM Tris-HCl, pH 7.5, 10 mM NaCl and 0.1 mM EDTA) was added 10 ml sodium nitrite (2 M) in 0.5 M sodium acetate buffer, pH 4.5. The solution was kept at room temperature for 2 hr and then chilled in ice for 1 hr. 5.0 ml of Na_2HPO_4 (2 M) was added to bring the pH back to 6.5. The crosslinked DNA thus obtained was

extensively dialysed against three changes (1000 ml each) TNE

Preparation of alkylated DNA and treatment with spermine and spermidine:

A 1 mg/ml solution of DNA in TNE was alkylated in sterile tubes by adding sufficient amount of N-methyl-N-nitrosourea (MeNu) or N-ethyl-N-nitrosourea (EtNu) to obtain a final concentration of 0.3 M each. The incubation was carried out for 1 hr at 37 C at the end of which the mixture was dialysed against three changes of 250 ml each of TNE for 12 hr at 4 C. The alkylated DNA was kept frozen and was thawed just before use.

In experiments where the effect of spermine and spermidine on the reaction of quercetin with DNA was studied, appropriate amounts of aqueous solutions of spermine and spermidine were added to obtain the final concentrations of 0.05 mM and 0.2 mM of spermine and spermidine, respectively.

(I) GENERATION OF OXYGEN FREE RADICAL BY METHYLGLYOXAL

(I) RESULTS

Fig. 1 shows the generation of superoxide anion ($O_2^{\cdot-}$) by MG in visible light. The increase in absorbance at 560 nm observed on reduction of NBT by $O_2^{\cdot-}$ is substantially reduced in the dark and is completely inhibited by superoxide dismutase (SOD), confirming the formation of this radical by MG. In Fig.2 is given the effect of MG concentrations on $O_2^{\cdot-}$ generation at increasing pH values. The maximum production of $O_2^{\cdot-}$ is seen at the alkaline pH of 9.5. However, at relatively high concentrations of MG (above 0.28 mM) a slight decrease in the formation of $O_2^{\cdot-}$ is seen at the pH values (7.8, 8.5, 9.5). Time course of the generation of $O_2^{\cdot-}$ by MG at pH values of 7.8, 8.5 and 9.5 is shown in Fig. 3. As can be seen, the rate of $O_2^{\cdot-}$ formation shows considerable enhancement at pH 9.5. Reproducibility of the above results was confirmed in several repeated experiments within 10% error. The $O_2^{\cdot-}$ formation as measured by increase in absorbance at 560 nm as an effect of time of reaction remains linear upto at least 40 min in both Fig. 1 and 3. The enhanced production of $O_2^{\cdot-}$ at elevated pH and the opposite inhibitory effect at higher MG concentrations is similar to the one observed in the case of xanthine oxidase catalysed generation of $O_2^{\cdot-}$ from xanthine or hypoxanthine (Fridovich, 1970). The formation of H_2O_2 by $O_2^{\cdot-}$ can occur by the following mechanisms. Addition of a second electron to $O_2^{\cdot-}$ gives the peroxide ion (O_2^{2-}) which has no unpaired electron and is not a radical. However, O_2^{2-} at pH around neutrality immediately protonates

Fig.1. Photogeneration of $O_2^{\cdot-}$ on illumination of MG in visible light.

The reactions were carried out as described in the "Methods". The concentration of MG in the reaction mixture was 0.18 mM. MG alone (●); MG + SOD (10 ug/ml) (*); MG alone incubated in dark (▲).

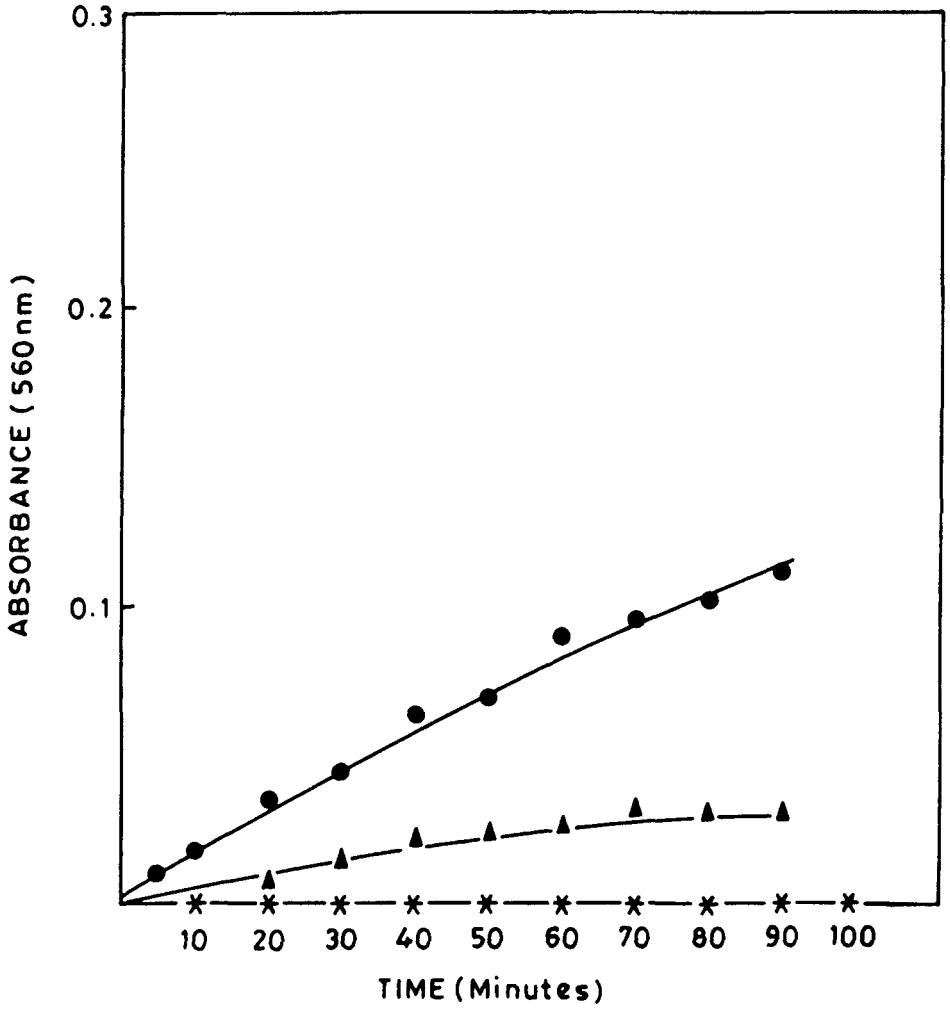

0.3
0.2
0.1
ABSORBANCE (560nm)
10
20
30
40
50
60
70
80
90
100
TIME (Minutes)

Fig.2. Effect of pH on the generation of $O_2^{\cdot-}$ as a function of MG concentrations.

pH 7.8 (*); pH 8.5 (▲); pH 9.5 (●). Details of reaction conditions are described in the "Methods."

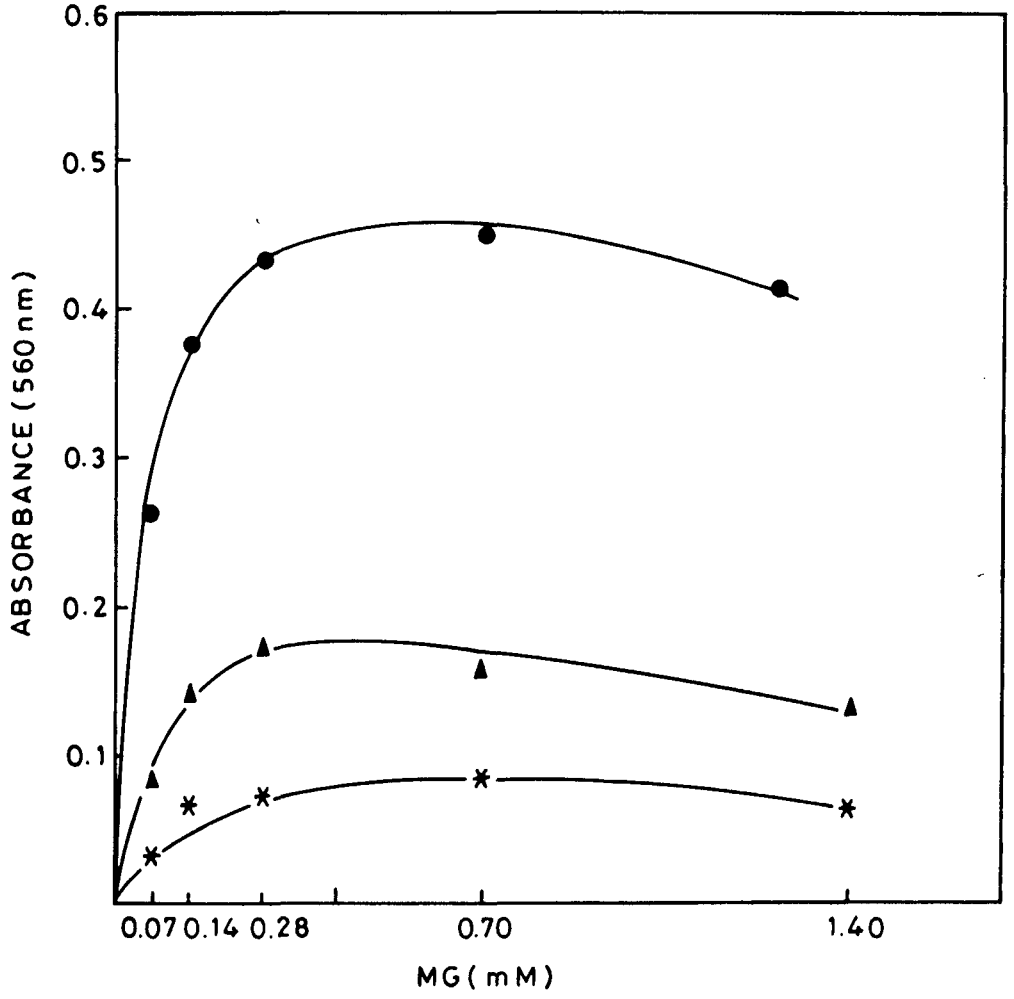
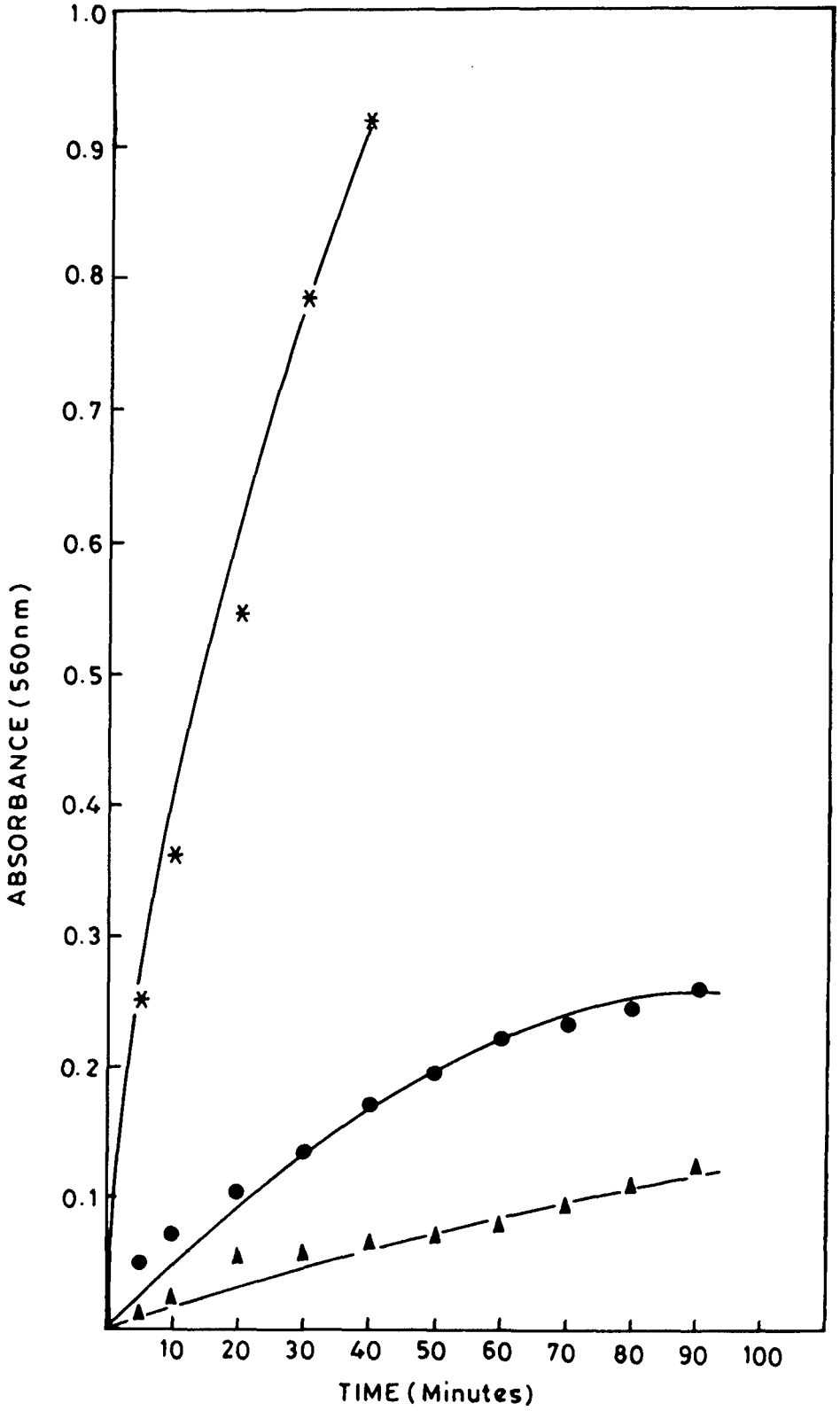

0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE (560 nm)
0.07 0.14 0.28
0.70
1.40
MG (mM)

Fig.3. Effect of pH on the generation of $O_2^{\cdot-}$ by MG as a function of illumination time.

MG was at 0.28 mM throughout. Other reaction conditions were the same as described in the "Methods." pH 7.8 (▲); pH 8.5 (●); pH 9.5 (*).


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE (560nm)
10
20
30
40
50
60
70
80
90
100
TIME (Minutes)

to give hydrogen peroxide (H_2O_2). In aqueous solutions $O_2^{\cdot-}$ also undergoes dismutation to form H_2O_2 and O_2 (Halliwell and Gutteridge, 1984).

$$2O_2^{\cdot-} + 2H^+ \text{ ---------- } H_2O_2 + O_2$$

The $O_2^{\cdot-}$ and H_2O_2 interact in the presence of iron salts to generate the OH in the so called Haber-Weiss reaction (Haber and Weiss, 1934; Beauchamp and Fridovich, 1970).

$$O_2^{\cdot-} + H_2O_2 \xrightarrow[\text{Catalyst}]{\text{Fe(III) salt}} O_2 + OH^{\cdot} + OH^-$$

In the following experiments we show that $OH^{\cdot}$ is also generated by MG presumably by the above mechanism. Table I shows the effect of increasing MG concentrations on the generation of $OH^{\cdot}$ as determined by the formation of hydroxylated salicylic acid. It is seen that the formation of $OH^{\cdot}$ increases upto 0.28 mM whereupon it gives a constant value on further increase in MG concentration. This observation is similar to the one observed with $O_2^{\cdot-}$ production (Fig. 2) suggesting the dependence of $OH^{\cdot}$ production on $O_2^{\cdot-}$ formation. Fig. 4 gives the generation of $OH^{\cdot}$ as a function of time. The formation of $OH^{\cdot}$ appears to be a slow process and gives a lag period of about 2 hrs. The effect of increasing pH (Table II) on $OH^{\cdot}$ generation shows a pattern similar to the one seen for $O_2^{\cdot-}$ formation (Fig.3) where maximum production is seen at pH values above 9.0.

In order to unequivocally demonstrate that $OH^{\cdot}$ is formed and follows the pathway of the Haber-Weiss reaction, the effect of quenchers of various active oxygen species was studied. The results

Table I. Formation of hydroxyl radicals as a function of MG concentrations.

Methylglyoxal (mM)	Hydroxylated products formed (n moles)
0.07	12.3
0.14	18.1
0.28	29.5
0.70	28.0
1.40	30.5

Reaction conditions are described in the 'Methods' and values shown are final reaction concentrations.

Fig.4. Formation of $\dot{OH}$ by MG as a function of illumination time.

The concentration of MG in the reaction mixture was 0.28 mM. Details of the reaction conditions are described in the "Methods."

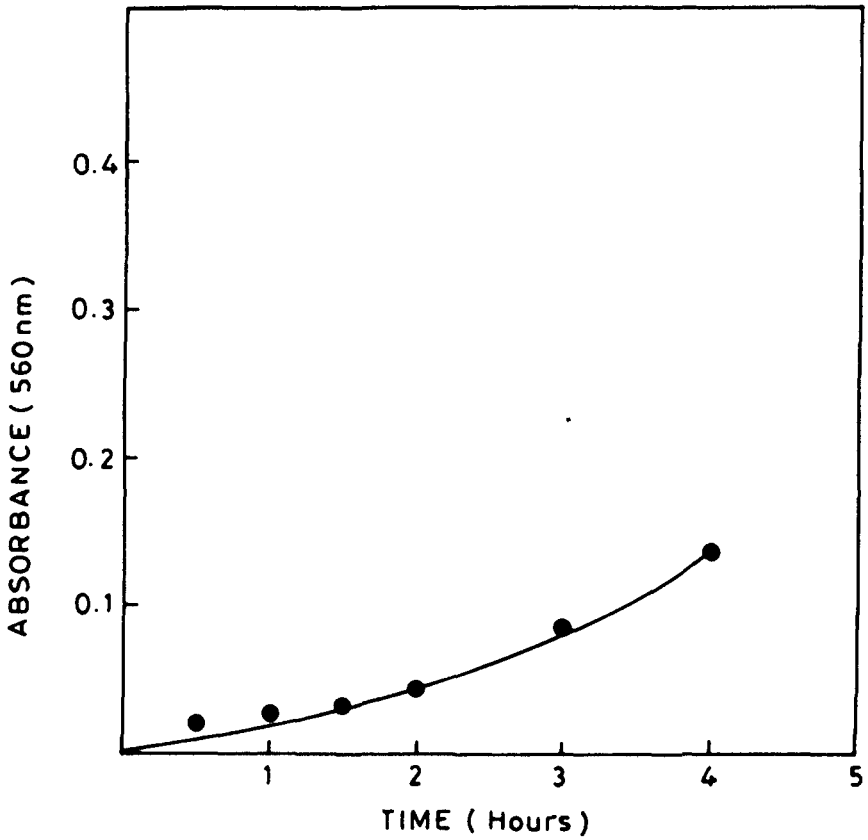

ABSORBANCE (560nm)
0.4
0.3
0.2
0.1
1
2
3
4
5
TIME (Hours)

Table II. Formation of hydroxyl radicals by MG as a function of pH.

pH	Hydroxylated products formed (n moles)
7.8	9.84
8.0	22.46
9.0	36.30
9.5	38.46

The final concentration of MG in the reaction mixture was 0.28 mM. Other reaction conditions were the same as given in 'Methods'.

Table III. Effect of oxygen radical quenchers on the generation of hydroxyl radicals by MG.

Quenchers	Hydroxylated products formed (n moles)	% inhibition
Control	15.9	0.0
Control + Catalase (25 ug/ml)	0.9	94.1
Control + SOD (20 ug/ml)	1.2	92.3
Control + mannitol (5 mM)	6.7	57.5
Control + albumin (50 ug/ml)	12.8	19.7
Control + sodium formate (5 mM)	7.4	53.6

The final concentration of MG in the reaction mixture was 0.28 mM. Concentrations of scavengers shown are the final reaction concentrations. Details of other reaction conditions are described in the 'Methods'. Control = Salicylate + MG + $FeCl_3$.

given in Table III shows that catalase and SOD show complete inhibition of $\dot{OH}$ formation confirming the involvement of $O_2^{\cdot-}$ and H_2O_2. The incomplete inhibition observed with mannitol and sodium formate, which are OH quenchers, is presumably due to the fact that these agents compete with the substrate i.e. salicylate for the $\dot{OH}$. In order to effectively trap $\dot{OH}$ by mannitol and sodium formate, very high concentrations (upto 50 mM) are required (Quinlan and Gutteridge, 1987). Of the various metal ions tested (Table IV), only Fe(III) and Co(II) were effective to any significant extent in generating $\dot{OH}$.

Table IV. Effect of metal ions on the generation of hydroxyl radicals by MG.

Metal ion (0.1 mM)	Hydroxylated product formed (n moles)
Fe(III)	7.4
Cu(II)	0.6
Co(II)	4.6
Ni(II)	0.0
Mn(II)	0.0

Details of reaction mixture are given in the 'Methods'.

(I) DISCUSSION

The $O_2^{\cdot-}$ dependent production of $OH^{\cdot}$ via Haber-Weiss reaction is well established (Rowley and Halliwell, 1982; McCord and Day, 1978). In these experiments the method used to detect OH involves the use of Fe(III)-EDTA complex to generate $OH^{\cdot}$ from $O_2^{\cdot-}$. The reaction sequence described by McCord and Day (1978) for $OH^{\cdot}$ formation is as follows.

$$O_2^{\cdot-} + \text{Fe(III)-EDTA} \text{ ------- } O_2 + \text{Fe(II)-EDTA}$$

$$\text{Fe(II)-EDTA} + H_2O_2 \text{ ------- } \text{Fe(III)-EDTA} + OH^- + OH^{\cdot}$$

$$O_2^{\cdot-} + H_2O_2 \text{ ------- } O_2 + OH^- + OH^{\cdot}$$

Under the reaction conditions used the $O_2^{\cdot-}$ reacts with the ferric ions. However, Halliwell (1975) has shown that it can also react with ferrous ions oxidizing it to ferric ion . In vivo the Haber-Weiss process is generally considered to occur when $O_2^{\cdot-}$ is generated in extracellular fluids devoid of catalase and SOD but contain a catalyst such as transferrin. These conditions are obtained in phagocyte mediated inflammatory processes (McCord, J.M., 1974; Salin,M.L. and McCord, J.M. 1975). In iron catalysed Haber-Weiss reaction the apparent role of $O_2^{\cdot-}$ is to reduce Fe(III) to Fe(II). However, it has been argue (Fee, 1982; Winterbourn, 1979) that this is not possible in vivo as the concentration of other biological reducing agents may be greater than that of $O_2^{\cdot-}$. Rowley and Halliwell (1982a and b) have however shown that NADH, NADPH and Glutathione or cysteine can interact with metal ions and H_2O_2 to increase $O_2^{\cdot-}$ formation under certain circumstances.

As already mentioned MG can arise in the cell by both enzymatic and non-enzymatic mechanisms from free trioses. It has been shown to be mutagenic in several systems and causes macromolecular crosslinks between DNA. and protein (Brambilla et al., 1985). The mechanism of mutagenic action of MG has not been investigated in sufficient detail. In this laboratory it was earlier demonstrated that the in vitro reaction of MG with DNA leads to the formation of strand breaks and interstrand crosslinks. The above results provide yet another mechanism by which MG can exert its mutagenic effect. The mutagenic/carcinogenic effect of various drugs such as quinone antibiotics has been ascribed to the generation of active oxygen species in addition to the binding of semiquinone radical to DNA (Nakayama et al, 1983). It has been observed that free radicals are generated by the metabolically formed proximate compounds of various chemical carcinogens such as benzo-a-pyrene and amino azo dyes (Nakayama et al., 1983).The free radicals thus formed are sufficiently reactive to bind to DNA (Nagata et al., 1980). This observation suggests a possible role of free radicals in carcinogenesis. Thus the ability of MG to generate active oxygen species may be important in relation to its mutagenic action. It also suggests that MG may act as a co-carcinogen in conjuntion with initiators of chemical carcinogenesis. It is generally considered that the reaction of $OH^{\cdot}$ with DNA is preceded by the formation of a complex between the radical producing chemical and DNA. This is followed by the production of $OH^{\cdot}$ at that particular site (Pryor, 1988). The $OH^{\cdot}$ is highly reactive

and it can only diffuse 5-10 molecular diameters before it can react (Pryor, 1988). Thus if it is not produced near DNA it will not react with it. Since MG must interact with DNA during the formation of strand breaks or crosslinks, the $\dot{OH}$ formation by it assumes additional significance.

(II) GENERATION OF OXYGEN FREE RADICALS AND DNA DEGRADATION BY QUERCETIN

(II) RESULTS

Recent work in this laboratory has shown that quercetin in the presence of Cu(II) leads to the degradation of DNA. Such degradation was demonstrated to require the participation of active oxygen species possibly the $\dot{O}H$ (Rahman et al., 1989a). As already described in the previous chapter the $\dot{O}H$ may arise in aqueous solution through the Haber-Weiss reaction which involves the participation of $O_2^{\cdot-}$ and H_2O_2. Fig.5 shows that $O_2^{\cdot-}$ is generated by quercetin in visible light. The increase in absorbance at 560 nm, observed on reduction of NBT, is significantly inhibited in dark and is not seen in the presence of SOD confirming the formation of $O_2^{\cdot-}$. In Fig.6 is given the rate of photogeneration of $O_2^{\cdot-}$ with increasing concentrations of quercetin. An increased production of $O_2^{\cdot-}$ is observed with increasing concentration of quercetin from 5-40 uM. However, at the highest concentration of 80 uM tested the rate of $O_2^{\cdot-}$ generation is reduced. This observation is similar to the one seen with MG (Fig.2) and in the case of xanthine oxidase-hypoxanthine generated $O_2^{\cdot-}$ (Fridovich, 1970). Hatcher and Bryan (1985) have shown that the auto-oxidation of quercetin is pH dependent and that it may be catalysed by $O_2^{\cdot-}$. However, in Fig.7 we demonstrated that the rate of $O_2^{\cdot-}$ photogeneration by quercetin is considerably enhanced as the pH is increased from 7.5 to 8.5.

The quercetin-Cu(II) mediated DNA degradation reaction is dependent upon molecular oxygen (Rahman et al., 1989a). On the basis of the action of various oxygen radical quenchers it was proposed

Fig.5. Photogeneration of $O_2^{\cdot-}$ on illumination of quercetin in visible light.

The photo-reactions were carried out as described in the "Methods". The concentration of quercetin in the reaction mixture was 20 uM.
quercetin alone (●); quercetin + SOD (10 ug/ml) (*); quercetin alone incubated in dark (▲)

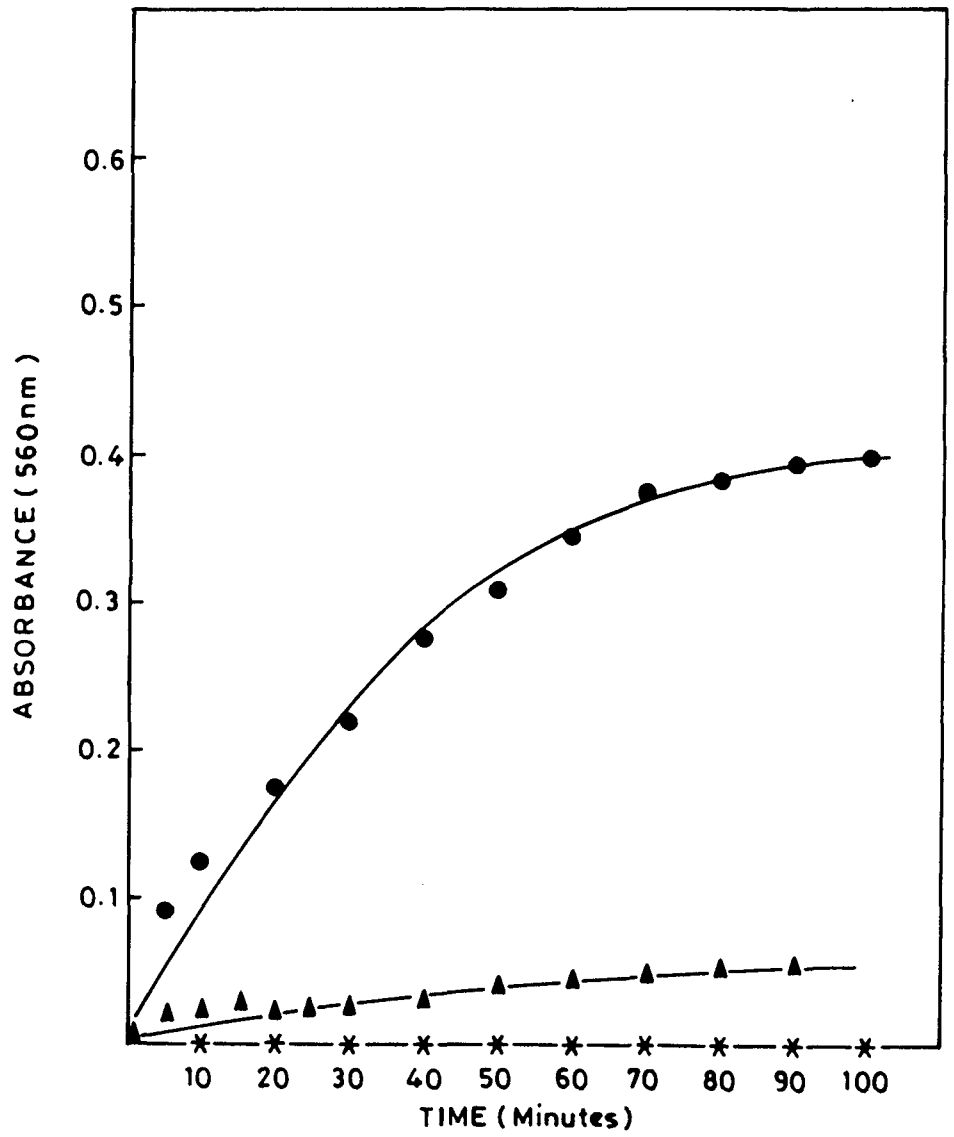

ABSORBANCE (560nm)
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
80
90
100
TIME (Minutes)

Fig.6. Effect of increasing concentrations of quercetin on the rate of $O_2^{\cdot -}$ generation.

5 uM (o); 10 uM (Δ); 20 uM (▲); 40 uM (●); 80 uM (*). Details of reaction conditions are described in the "Methods".

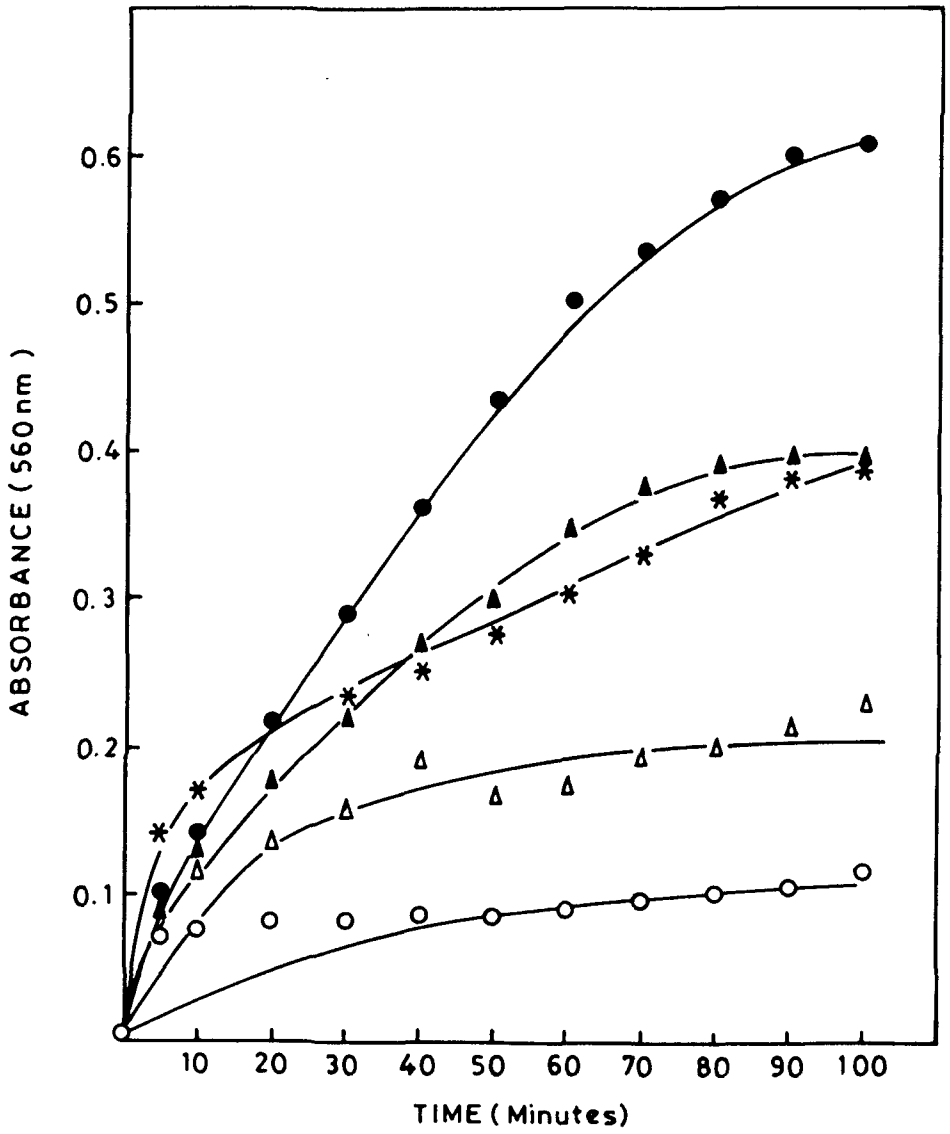

ABSORBANCE (560 nm)
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
80
90
100
TIME (Minutes)

Fig.7. Effect of pH on the generation of $O_2^{\cdot -}$ by quercetin as a function of illumination time.

The concentration of quercetin in the reaction mixture was 60 uM. Other reaction condition were the same as described in the "Methods."
pH 7.5 (o); pH 8.0 (▲); pH 8.5 (●).

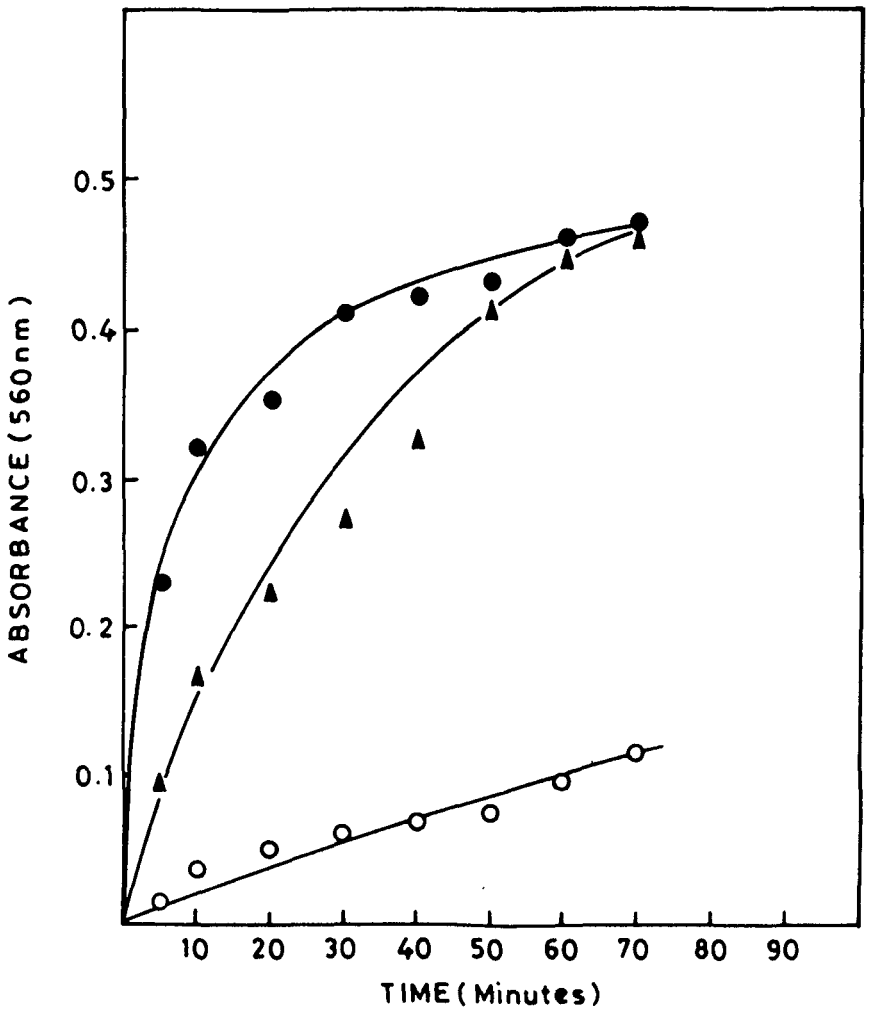

ABSORBANCE (560nm)
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
80
90
TIME (Minutes)

Table V. Formation of hydroxyl radicals with increasing concentrations of quercetin

Quercetin (uM)	Hydroxylated products formed (n moles)
5	13.53
10	18.46
20	32.92
40	47.38
80	98.15

Reaction conditions are given in the 'Methods' and values shown are final reaction concentrations.

that the proximate DNA breaking oxygen species is the $OH^{\cdot}$ radical. Table V gives the formation of $OH^{\cdot}$ with increasing concentrations of quercetin as measured by hydroxylation of salicylic acid method of Richmond et al (1981). An increase in the degree of $OH^{\cdot}$ generation is seen with quercetin concentrations from 5-80 uM. As described in the case of MG this method of $OH^{\cdot}$ detection involves, the use of Fe(III) or Cu(II)-EDTA complex to generate $OH^{\cdot}$ from $O_2^{\cdot -}$ and H_2O_2 (Richmond et al., 1981; McCord and Day, 1978). In order to test whether the Haber-Weiss pathway for $OH^{\cdot}$ is operative in this system, the effect of various oxygen radical scavengers was studied and is shown in Table VI. Significant inhibition of $OH^{\cdot}$ production is seen with SOD and catalase which are $O_2^{\cdot -}$ and H_2O_2 scavengers, respectively. $OH^{\cdot}$ quenchers sodium benzoate, mannitol and thiourea also show substantial inhibition indicating the involvement of these radicals.

In order to directly demonstrate the degradation of DNA by quercetin via $OH^{\cdot}$, fluorescent intensity of TBA adduct formed on DNA degradation was measured. This method depends on the formation of a chromogen on DNA damage by $OH^{\cdot}$ which forms an adduct with TBA and which fluoresces at 553 nm (Quinlan and Gutteridge, 1987). The fluorescent characteristics of the TBA adducts formed from products released from DNA deoxyribose and quercetin are shown in Fig.8b. As can be seen the spectrum is indistinguishable from the TBA adduct formed by 1-10 phenanthroline (Fig.8a) which is known to degrade DNA by $OH^{\cdot}$ in the presence of Cu(II) (Gutteridge, 1984). It is observed that some TBA-reactive material is formed when quercetin is treated with Cu(II) in

Table VI. Formation of hydroxyl radicals by quercetin in the presence of oxygen radical quenchers

Quenchers	Hydroxylated products formed (n moles)	% inhibition
Control	62.0	0.0
Control + SOD (0.1 mg/ml)	30.0	51.6
Control + catalase (0.1 mg/ml)	26.0	58.0
Control + mannitol (50 mM)	26.0	58.0
Control + sodium benzoate (50 mM)	9.3	85.0
Control + thiourea (10 mM)	4.0	93.5

Concentrations shown are the final reaction concentrations. Concentration of quercetin in the reaction mixture was 60 uM. Details of other reaction conditions are given in the 'Methods'.
Control = Salicylate + quercetin + Cu(II)

Fig.8a. Fluorescent scan of the TBA adducts formed from damage to DNA by copper-phenanthroline complex (taken as standard).

Fluorescent scan was made at 532 nm excitation and 553 nm emission. The reaction conditions leading to the formation of TBA-reactivity are fully described in the "Methods."

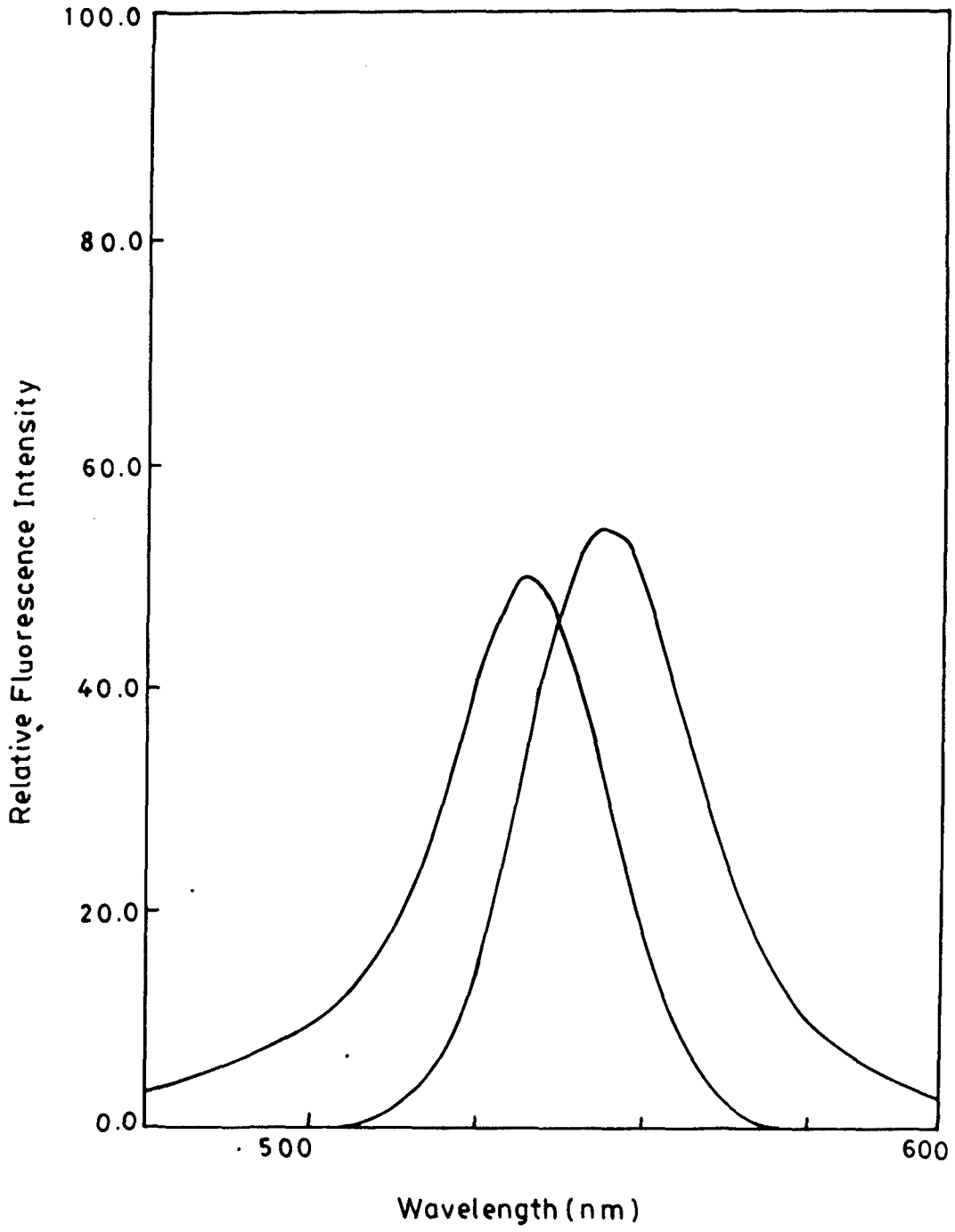
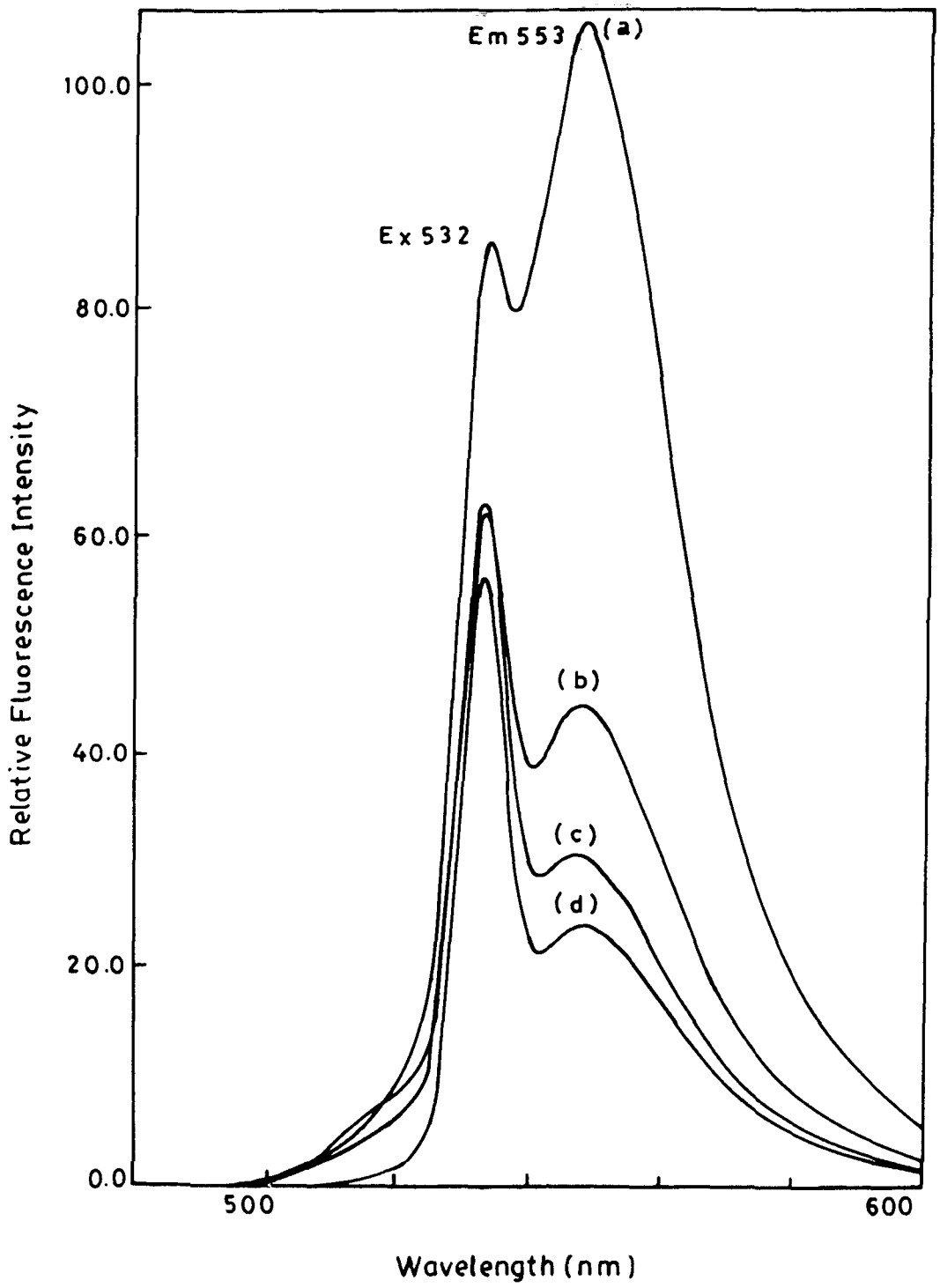

100.0
80.0
60.0
40.0
20.0
0.0
Relative Fluorescence Intensity
500
600
Wavelength (nm)

Fig.8b. Fluoresecent scans of TBA adducts formed from damage to DNA induced by quercetin-Cu(II) complex.

(a) Cu(II)-quercetin damage to DNA; (b) Cu(II) + DNA; (c) quercetin + DNA; (d) Cu(II) + quercetin. The fluorescent scans were made as described in Fig.8a. Details of the reaction conditions leading to the formation of TBA-reactivity are fully described in the "Methods."


Em 553
(a)
Ex 532
(b)
(c)
(d)
100.0
80.0
60.0
40.0
20.0
0.0
500
600
Relative Fluorescence Intensity
Wavelength (nm)

the absence of DNA (Fig.8,curve d). The production of TBA-reactive material further increases when DNA is incubated with quercetin alone (curve c) and when DNA is incubated with Cu(II) alone (curve b). However, when linear duplex DNA is incubated with both Cu(II) and quercetin they were able to bring about the maximum release of TBA-reactive material as indicated by the sharp enhancement in fluorescence at 553 nm (curve a). This confirms our previous results which suggested that DNA is degraded by $OH^{\cdot}$ in the presence of quercetin and Cu(II) (Rahman et al., 1989a).

The release of TBA-reactive material from DNA by quercetin-Cu(II) was also measured as a function of quercetin and Cu(II) concentrations. The results are given in Table VII and VIII. It may be seen that the $OH^{\cdot}$ formation increases with increasing quercetin concentrations upto 150 uM after which it becomes constant. With increasing Cu(II) concentration a progressive increase in the rate of $OH^{\cdot}$ formation is seen. The ability of other metal ions to release TBA-reactive material was also examined (Table IX). None of the several metal ions except Cu(II) tested at a concentrations of 0.1 mM induced the release of TBA-reactive material from DNA in the presence of quercetin.

Fluorescence changes that occur when quercetin binds to variously modified DNA:

It is generally believed that $OH^{\cdot}$ radical reactions with DNA are preceded by the formation of a drug-DNA complex and that the cleavage of DNA occurs close to the site of complex formation (Pryor, 1988). In order to determine whether quercetin binds to DNA at specific sites,

Table VII. Formation of TBA-reactive material with increasing concentrations of quercetin as measured by relative fluorescence intensity.

Quercetin (uM)	Relative fluorescence intensity units (RFI) Ex 532 nm and Em 553 nm
25	15.8
50	20.2
100	23.0
150	27.0
200	26.3
Blank [DNA + Cu(II)]	10.2

Concentration shown are the final reaction concentrations. Details of the reaction conditions are given in the 'Methods'.

Table VIII. Formation of TBA-reactive material with increasing Cu(II) concentrations as measured by relative fluorescence intensity.

Cu(II) (uM)	Relative fluorescence intensity units (RFI) Ex 532 nm and Em 553 nm
25	23.2
50	27.4
100	29.6
150	28.3
200	27.1
Blank [DNA + quercetin]	7.0

Concentration shown are final reaction concentrations. Concentration of quercetin in the reaction mixture was 60 uM. Details of the reaction mixture are given in the methods.

Table IX . Release of TBA-reactive material following damage to DNA by quercetin and transition metal ions

Metal ion (0.1 mM)	Relative fluorescence intensity units (RFI) Ex 532 nm and Em 553 nm		
	DNA + Metal ion	Quercetin + Metal ion	DNA + Quercetin + Metal ion
Cu(II)	30.5	18.0	51.5
Fe(III)	29.2	16.0	34.0
Mn(II)	25.7	17.0	27.5
Co(II)	21.0	16.0	23.0
Ni(II)	19.0	13.0	23.0

Details of the reaction conditions are as described in the 'Methods'.

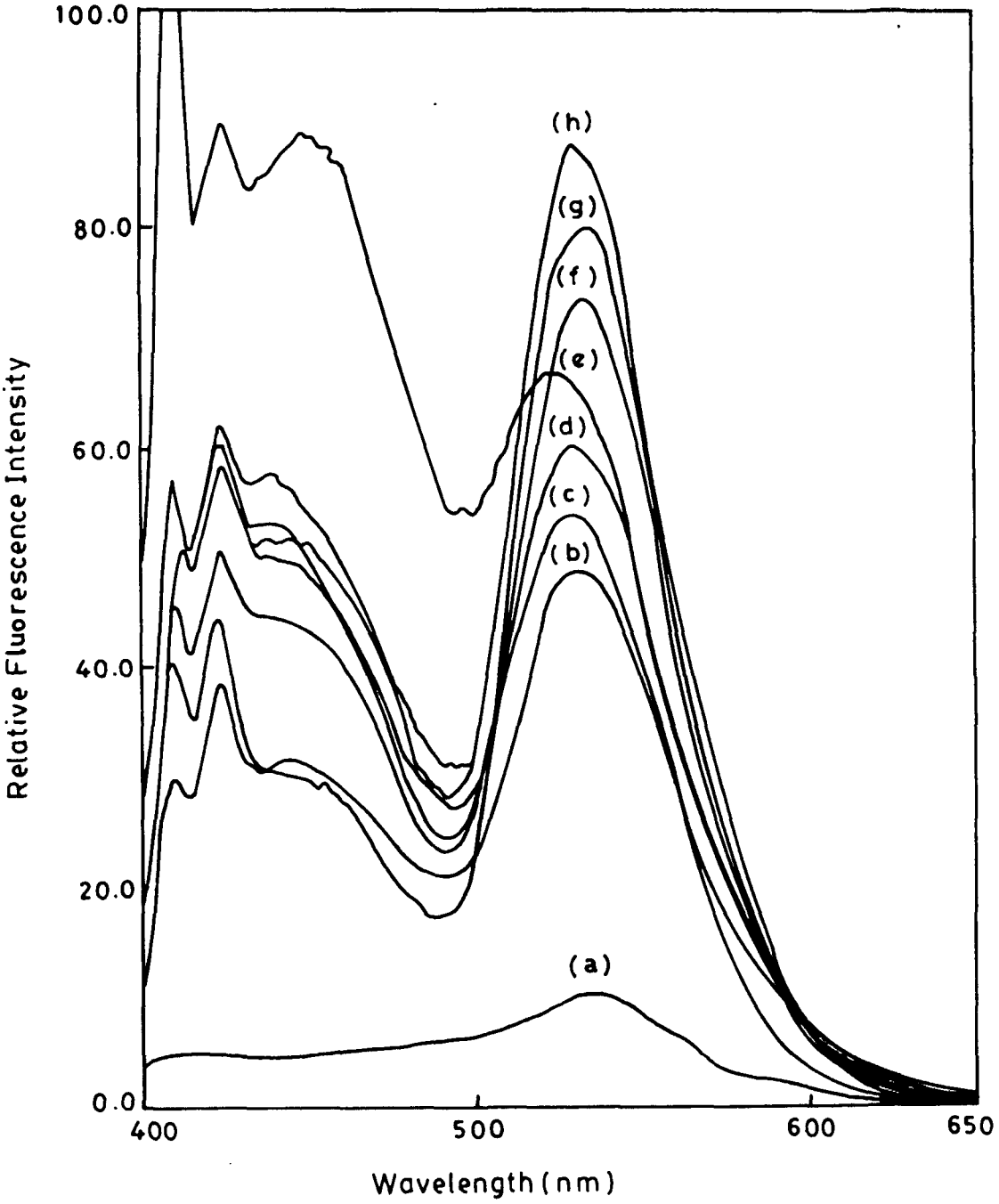
the DNA was first treated with various well established DNA binding and DNA reacting drugs before incubation with quercetin and measurement of fluoresence spectra. In case quercetin binds to any of the sites with which such drugs also react then a change in the fluorescence spectrum of quercetin-DNA complex should be observed. The results given in Fig.9 show that quercetin alone does not exhibit any detectable fluorescence (curve a). However, upon binding with DNA a remarkable enhancement in the fluorescence of quercetin is observed. The enhancement was maximum in the case of denatured DNA (curve h), whereas native and crosslinked DNA showed almost the same degree of enhancement (curve c and b). These results, therefore, suggest that quercetin does not intercalate in the duplex DNA. N-methyl-N-nitrosourea (MeNu) and N-ethyl-N-nitrosourea (EtNu) are DNA alkylating agents and predominantly alkylate DNA bases and DNA phosphates, respectively (Sun and Singer, 1975). Alkylated DNA on binding with quercetin show further enhancement in the fluorescence (curve d and e). Spermine and spermidine preferentially bind to DNA through surface interactions (Rajalakshmi et al., 1978). DNA treated with both these compounds also show increased fluorescence after reaction with quercetin (curve g and f). Possibly, these results are accounted for partial denaturation of DNA during treatment with these drug binding agents. These results therefore do not provide any information on the specificity of quercetin interaction with DNA.

Restriction analysis of quercetin-Cu(II) treated DNA :

In order to study the specificity of DNA breakage induced by quercetin

Fig.9. Fluorescence emission spectra of quercetin in presence of variously modified DNA.

The quercetin concentration was 25 uM and the DNA bp/quercetin molar ratio was 60:1 throughout. The excitation wavelength was set at 365 nm and the emission wavelength at 530 nm. (a) quercetin alone; (b) crosslinked DNA; (c) native DNA; (d) MeNu alkylated DNA; (e) EtNu alkylated DNA; (f) spermidine mixed DNA; (g) Spermine mixed DNA; (h) denatured DNA.


100.0
80.0
60.0
40.0
20.0
0.0
Relative Fluorescence Intensity
(h)
(g)
(f)
(e)
(d)
(c)
(b)
(a)
400
500
600
650
Wavelength (nm)

and Cu(II), protection of cleavage sites from the action of various restriction enzymes was used. There are several reports on the use of restriction enzymes on the study of structural alterations of DNA caused by DNA specific drugs (Nosikov et al., 1976; Goppelt et al., 1981). Restriction analysis of lambda phage DNA reacted with quercetin and Cu(II) was done by selecting enzymes having exclusively or predominantly GC or AT base pairs in their recognition sequences. Fig. 10,11 and 12 show experiments where DNA was treated with increasing concentrations of quercetin in presence of 0.1 mM Cu(II) and digested with DraI (TTT/AAA), SmaI (CCC/GGG) and EcoRI (GA/ATTC), respectively. The samples treated with quercetin upto 50 uM concentration were cleaved with DraI giving a pattern similar to that with untreated DNA (Lanes 2 and 4). However, substantial inhibition of cleavage occured at 100-200 uM quercetin (Lanes 5 and 6). Similarly complete inhibition of cleavage was observed with SmaI at 200 uM quercetin concentration (Lane 6). In another experiment maximum inhibition of EcoRI was produced with 100 uM quercetin (Lane 6). These results thus indicate that there is no specificity involved in the DNA breakage induced by quercetin and Cu(II)

Effect of riboflavin on the quercetin-Cu(II) mediated DNA degradation: Since the photo-oxidation of quercetin, which is very slow, is considerably accelerated by riboflavin via $O_2^{\cdot -}$ (Takahama, 1985), it was of obvious interest to compare the rate of quercetin-Cu(II) mediated DNA degradation in the absence (Fig.13) and presence of riboflavin (Fig.14). The presence of riboflavin caused a 4-fold increase in the

Fig.10. Effect of increasing concentrations of quercetin quercetin on DraI digestion of lambda phage DNA in the presence of Cu (II).

Lane 1: DNA alone, Lane 2: 100 uM Cu(II), Lanes: 3-6: 25, 50, 100 and 200 uM quercetin in the presence of 100 uM Cu(II). All samples contained 0.5 ug DNA and the incubation was carried out at 37 C for 1 hr. The treated samples were then subjected to digestion by DraI following removal of unreacted quercetin by dialysis as described in the "Methods."

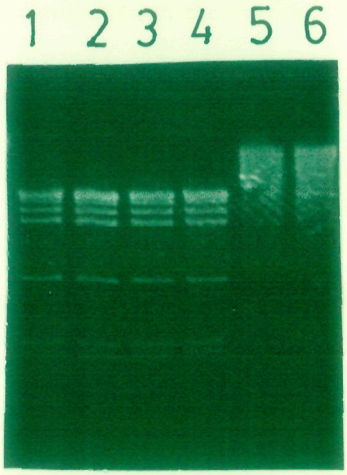

1 2 3 4 5 6

Fig.11. Effect of increasing concentrations of quercetin on SmaI digestion of lambda phage DNA in the presence of Cu(II).

Lane 1: DNA alone, Lane 2: 100 uM Cu(II), Lanes 3-6: 25, 50, 100 and 200 uM quercetin in the presence of 100 uM Cu(II). All samples contained 0.5 ug DNA and the incubation was carried out at 37 C for 1 hr. The treated samples were then subjected to digestion by SmaI following removal of unreacted quercetin by dialysis as described in the "Methods."

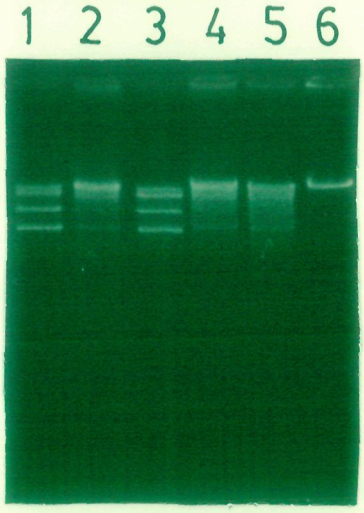

1 2 3 4 5 6

Fig.12. Effect of increasing concentrations of quercetin on EcoRI digestion of lambda phage DNA in the presence of Cu(II).

Lane 1: DNA alone, Lane 2: 100 uM Cu(II), Lanes 3-6:12.5, 25, 50, 100 uM quercetin in the presence of Cu(II).Other reaction conditions were the same as described in Fig.10.

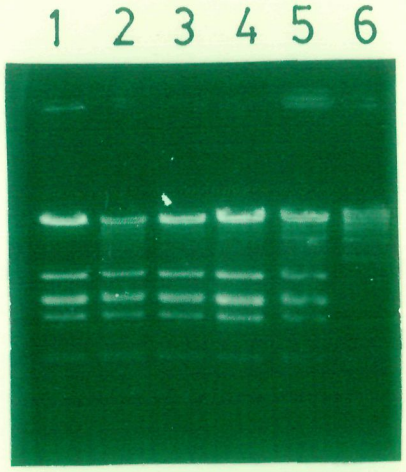

1 2 3 4 5 6

Fig.13. Agarose gel electrophoretic pattern of ethidium bromide stained pSK 275 DNA after treatment with quercetin and Cu(II).

DNA migrated from top to bottom in the order of decreasing distance of form I (supercoiled covalently closed circular DNA), form III (linear duplex DNA) and form II (relaxed circular DNA). The reaction mixture containing 0.5 ug pSK 275 DNA, 0.1 mM quercetin and Cu(II) each in 10 mM Tris-HCl buffer, pH 7.5, were incubated at room temperature in visible light for the periods indicated. Lanes 1-7: 0, 5, 10, 30, 60, 120, and 180 min.

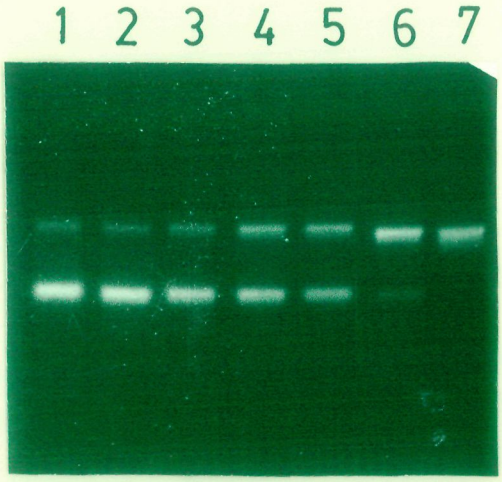
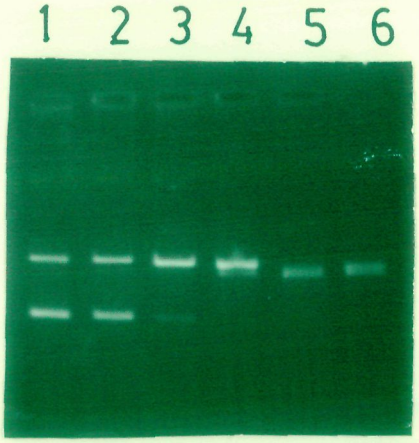

1 2 3 4 5 6 7

Fig.14. Kinetic behaviour of DNA breakage induced by quercetin and Cu(II) in presence of riboflavin.

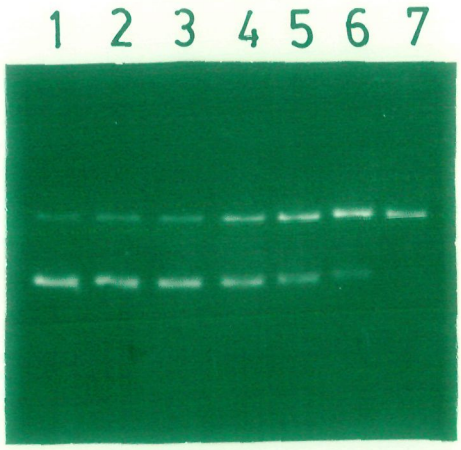
The reaction conditions were exactly the same as described in Fig.13. The only difference was that it was carried out in the presence of 5 uM riboflavin. Lanes 1-6: 0, 10, 30, 60, 120 and 180 min.


1 2 3 4 5 6

rate of DNA breakage reaction of quercetin and Cu(II). In the presence of riboflavin, form I (covalently closed supercoiled) DNA was almost completely converted to form II (relaxed circular) DNA in 30 min (Fig.14,Lane 3). The same was observed in 2 hr when riboflavin was not present (Fig.13,Lane 7). Since riboflavin alone has been shown to degrade DNA (Korycha-Dahl and Richardson, 1980), another experiment was performed in which DNA cleavage by riboflavin alone was examined as a function of time (Fig 15). The results indicate that the conversion of form I to form II DNA starts only after 30 min and reaches completion in 3 hr (Lanes 4 and 7). Thus the accelerated photo-oxidation of quercetin by riboflavin correlates with the increased rate of DNA breakage by quercetin and Cu(II) in the presence of riboflavin.

Fig.15. Kinetic behaviour of DNA damage induced by riboflavin.

Reaction mixtures containing 0.5 ug pSK 275 DNA and 5 uM riboflavin in 10 mM Tris-HCl buffer, pH 7.5 were incubated at room temperature in visible light for the periods indicated. Lanes 1-7; 0, 5, 10, 30, 60, 120 and 180 min.


1 2 3 4 5 6 7

(II) DISCUSSION

The results described above can be summarised as follows: quercetin generates $O_2^{\cdot-}$ in aqueous solution which is stimulated in visible light. The production of $O_2^{\cdot-}$ can lead to the formation of $OH^{\cdot}$ through the Haber-Weiss reaction which involves the participation of H_2O_2 and a metal ion. This was established by the quenching effect of SOD and catalase, mannitol, sodium benzoate and thiourea on $OH^{\cdot}$ production. Quercetin in the presence of Cu(II) can also lead to degradation of DNA as measured by the formation of TBA-reactive material. The DNA degradation reaction does not appear to involve any sequence specificity. This was determined by restriction analysis of quercetin-Cu(II) degraded DNA and by measurement of changes in the fluorescence of quercetin after its reaction with variously modified DNAs. The DNA degradation of quercetin-Cu(II) is considerably enhanced in the presence of riboflavin which is an established photosensitizer and produces $O_2^{\cdot-}$ in visible light.

As mentioned in the "Introduction" the mutagenic activity of quercetin is substantially increased by the rat liver S9 preparation. The results of Rahman et al. (1989a) and the experiments described above would suggest that the mutagenic action of quercetin is related to its capacity to degrade cellular DNA in the presence of metal ion and molecular oxygen. Hatcher and Bryan (1985) have studied several factors which alter the mutagenic activity of quercetin such as metal ion, anti-oxidants and pH. According to these authors metal ions such

as Cu(II) and Fe(III) are inhibitory in quercetin mediated mutagenisis of S. typhimurium. SOD enhances the mutagenic activity of quercetin by scavenging $O_2^{\cdot-}$ and thus inhibiting the auto-oxidation of quercrtin. These results were interpreted by suggesting that quercetin may oxidize to a ortho-quinone form, either through a metal or $O_2^{\cdot-}$catalysed reaction, and that this ortho-quinone cannot enter the cell due to ionization of hydroxyls. This is due to the fact that an ionized molecule cannot cross the cell membrane as readily as a non-ionized one. However, once quercetin has entered the cell both metal as well as $O_2^{\cdot-}$ should have a stimulatory effect on its mutagenicity. Thus our findings are not in contradiction of the results of Hatcher and Bryan (1985). Takahama (1985) has shown that the photo-oxidation of quercetin is considerably enhanced by the photo-sensitizer riboflavin. Such photo-oxidation presumably occurs through the formation of $O_2^{\cdot-}$. Our results (Fig.15) show that at concentration of riboflavin which does not show any significant degradation of DNA, quercetin-Cu(II) mediated DNA breakage is considerably enhanced. Since photo-sensitized riboflavin also generates $O_2^{\cdot-}$, it is indicated that $O_2^{\cdot-}$ mediated oxidation of quercetin or DNA-quercetin-Cu(II) is involved in the DNA degradation reaction. This is of significance as quercetin may also be oxidized by electron transfer from excited quercetin to molecular oxygen.

BIBLIOGRAPHY

ALDERSON,T. AND CLARK,A.M.(1966) Nature, 210, 593.

AMBROSE,A.M., ROBBINS,D.J. AND DE.EDS.F. (1952) J.Am.Pharm.Assoc., 40, 119.

AMES,B.N. (1972) In: Mutagenic Effects of Environmental Contaminants, Sulton, H.E. and Harris, M.I., Eds. (Academic Press, New York) 57.

AMES,B.N.(1979) Science, 204, 587.

AMES,B.N.(1983) Science, 221, 1256.

AMES,B.N., CATHCART,R., SCHWIERS,E. AND HOCHSTEIN,P.(1981) Proc.Natl. Acad.Sci.USA, 78, 6858.

BATZINGER,R.P., OU,S-YL. AND BUEDING,E.(1977) Science, 198, 944.

BEAUCHAMP,C.O. AND FRIDOVICH,I. (1970) J.Biol.Chem. 245, 4641.

BECKMAN,C., ROY,R.M. AND SPROULE,A.(1982) Mutat.Res., 105, 73.

BISCHOFF,F.(1969) Adv.Lipid Res., 7, 165.

BJELDANES,L.F. AND CHANG,G.W.(1977) Science, 197, 577.

BONSIGNORE,A., LEONCINI,G., RICCI,D. AND SIRI,A.(1976) Ital.J.Biochem. (Engl.Ed.), 22, 131.

BORCHERT,P., WISLOCKI,P.G., MILLER,J.A. AND MILLER, E.C.(1973) Cancer Res., 33, 1323.

BOYD,J.N.,BABISH,J.G. AND STOEWSAND,G.S.(1982) Food Chem.Toxicol., 20, 47.

BRACKE,M.E., De PESTEL, G., CASTRONOVO, V., VYNCKE, B., FIODART, J.M., VAKAET,L.C.A. AND MAREEL,M.M. (1987) In: 'Plant Flavonoids in Biology and Medicine: biochemical, cellular and medicinal properties', Eds. Cody, V., Middleton, E., Harborne, J.B. and Beretz, A., (Alan R. Liss, New York) 45.

BRAMBILLA,G., SCIABA,L., FINOLLO,R., BASSI,A.M.,FERRO,M. AND MARINARI, U.M. (1984) Cell Biochem. Funct., 2, 243.

BRAMBILLA,G., SCIABA,L., FAGGIN,P., FINOLLO,R., BASSI,A.M., FERRO,M. AND MARINARI, U.M.(1985) Carcinogenesis, 6, 683.

BROWN,J.P.(1980) Mutat.Res., 75, 243.

BUETTNER,G.R., OBERLY,L.W. AND CHAN LEUTHAVSER,S.W.H.(1978) Photochem. Photobiol., 28, 693.

CAJELLI,E., CANONERO,R.,MARTELLI,A. AND BRAMBILLA,G.(1987) Mutat.Res., 190, 47.

CAMPBELL,M.A. AND FANTEL,A.G.(1983) Life Sci., 32, 2641.

CARMELLA,S.G.,LAVOIE,E.J. AND HECHT,S.S.(1982) Food Chem.Toxicol., 20, 587.

CHRISEY,L.A., SHAHIDI BONJAR,G.H. AND HECHT,S.M.(1988) J.Am.Chem.Soc., 110, 644.

CLARK,A.M.(1960) Z.Vererbungls., 91, 74.

CLARK,A.M.(1982) In:Environmental Mutagenesis,Carcinogenesis and Plant Biology, Klekowsky,E.J.Jr., Ed. (Praeger, New York) 1, 1977.

CONCON,J.M., NEWBURG,D.S. AND SWERCZEK,T.W.(1979) Nutr.Cancer, 1, 22.

COOPER,R.A.(1974) Eur.J.Biochem., 44, 81.

COOPER,R.A. AND ANDERSON,A.(1970) FEBS Lett., 11, 273

DEMOPOULOS,H.B., PIETRONIGRO,D.D., FLAMM,E.S. AND SELIGMAN,M.L.(1980) J.Environ.Pathol.Toxicol., 3, 273.

DIANZANI,M.U.(1979) In:Submolecular Biology and Cancer Ciba Foundation Series 67, Amsterdam: Excerpta Medica, 245.

DIANZANI,M.U.,BACCINO,F.M. AND TESSITORE,L(1978) IRCS Med.Sci.,6, 274.

DIANZANI,M.U., BACCINO,F.M., MESSINA,M., CECCHINI,G., BARRERA,G. AND TESSITORE,L.(1980) IRCS Med.Sci., 8, 119.

DOLL,R. AND PETO,R.(1981) J.Natl.Cancer Inst., 66, 1192.

DRINKWATER,N.R.,MILLER,E.C. AND MILLER,J.A.(1976) J.Natl.Cancer Inst., 57, 1323.

DUNNICK,J.K., PREJEAN,J.D., HASEMAN,J., THOMPSON,R.B., GILES,H.D. AND McCONNEL,E.E.(1982) Fundam.Appl.Toxicol., 2, 114.

DURAN,N., FARIA OLIVERIA,O.M.M., HUAN,M. AND CILENTO,G.(1977) J.Chem. Soc.Chem.Commun.,442

ELLIOTT,W.H.(1960) Nature, 185, 467.

EVANS, J.G., BUTTERWORTH, K.R., GAUNT, I.F. AND GRASSO, P.(1977) Food Cosmet.Toxicol., 15, 523.

FABRO,S.(1982) Reprod.Toxicol., 1, 2.

FAGGIN,P., BASSI,A.M., FINOLLO,R. AND BRAMBILLA,G. (1985) Mutat.Res., 144, 189.

FEE,J.A. (1982) In: Proc. 3rd Int. Cong. Oxidases and Related Redox Systems, Morrison,M. et al., Eds. (Pergamon, Oxford), 1981.

FENAROLI, G. (1971) In: Handbook of Flavor Ingredients, Furia, T.E., Bellanco,N., Eds., Chemical Rubber Co., Cleveland, Ohio.

FENSELAU,A. AND LONG,C. (1976) Eur.J.Cancer, 12, 859.

FERRALI,M., FULCERI,R., BENEDETTI,A. AND COMPORTI,M.(1980) Res.Commun. Chem.Pathol.Pharmacol., 30, 99.

FINK,D.J. AND KRITCHEVSKY,D. (1981) Cancer Res., 41, 3677.

FISCHER,S.M., MILLS,G.D. AND SLAGE,T.J. (1982) Carcinogenesis,3, 1243.

FISHBEIN,L. (1983) Proceedings of the XV International Congress of Genetics, 3, 3.

FISHBEIN,L. FLAMMA,W.G. AND FALK,H.L. (1970) Chemical Mutagens (Academic Press, New York).

FLOHE,L. (1982) In: Free Radicals in Biology, Pryor,W.A. Ed. (Academic Press, New York) 5, 223.

FUJITA,Y., WAKABAYASHI,K., NAGAO,M. AND SUGIMURA,T. (1985) Mutat.Res., 142, 145.

FURIHATA,C., SATO,Y., MATSUSHIMA,T. AND TATEMATSU,M. (1985) Carcinogenesis, 6, 91.

FRIDOVICH,I.,(1970) J.Biol.Chem.,245,4053

GABRIEL,L.,VIRONDA,N., BONELLI,G. AND MIGLIETTA,A.(1985) Cell Biochem. Funct., 3, 9.

GENSLER,H.L. AND BERNSTEIN,H. (1981) Q.Rev.Biol., 6, 279.

GOPPLET,M., LONGOWSKI,J., PINGOND,A.,HAUPT,W., URBANK,C., MAYER,M. AND G.MAASS (1981) Nucleic Acids Res., 9, 6115.

GREGG,T.CH. (1968) Biochim.Biophys.Acta, 155, 271.

GRIESEBACH,H. AND EBEL,J. (1978) Angew.Chem.Int. (Engl.Ed.) 17, 635.

GUENTHER,E. AND ALTHAUSEN,D. (1949) The Essential Oils (Van Nostrand, New York), 2, 499.

GUTTERIDGE,J.M.C. (1984) Biochem.J., 218, 983.

HABER,F. AND WEISS,J. (1934) Proc. R. Soc. London Ser. 147, 332.

HALLIWELL,B.(1975) FEBS Lett. 56, 34.

HALLIWELL,B. AND GUTTERIDGE,J.M.C (1984) Biochem,J., 219, 1.

HARMAN,D. (1981) Proc.Natl.Acad.Sci.USA, 78, 7124.

HAROZ,R.K. AND THOMASSAN,J. (1980) Toxicol.Lett. (Suppl). 6, 72.

HARTMAN,P.E. (1982) In: Clinical Mutagen, de Serres,F.J. and Hollaender, Eds. (Plenum, New York) 7, 211.

HATCH,G.E., GARDNER,D.E. AND MENZEL,D.B. (1980) Environ.Res., 23, 121.

HATCHER,J.F. AND BRYAN,G.T. (1985) Mutat.Res., 148, 13.

HECKER,E. (1981) J.Cancer Res.Clin.Oncol., 99, 103.

HENDRICKS, J.D., SINNHUBER, R.O., LOVELAND, P.M., PAWLOWSKI, N.E. AND NIXON, J.E. (1980) Science, 208, 309. .

HERMANN,K. (1976) J. Food Sci. Technol., 11, 433.

HIRONO,I. (1981) CRC Crit Rev.Toxicol., 8, 253.

HIRONO.I., MORI,H., YAMADA,K.,HAGA,M., TATEMATSU,H. AND KANIE,S.(1977) J.Natl.Cancer Inst., 58, 1155.

HIRONO,I., UENO,I., HOSAKA,S., TAKANASHI, H., MATSUSHIMA,T., SUGIMURA, T. AND NATORI,S.(1981) Cancer Lett., 13, 15.

HOCHSTEIN,P. AND JAIN,S.K. (1981) Fed.Proc.Fed.Am.Soc.Exp.Biol., 40, 183.

IMAI,H., WERTHESSEN,N.T., SUBRAMANIAM, V., LEUESNE, P.W., SOLOWAY,A.H. AND KANISAWA,M. (1980) Science, 207, 651.

IVIE,G.(1978) In: Effects of Poisonous Plants on Livestock, Keeler,R., Vankampen,K. and James,L., Eds. (Academic Press, New York), 475.

IVIE,G.W., HOLT,D.L. AND IVEY,M.C. (1981) Science, 213, 909.

JADHAV,S.J.,SHARMA,R.P. AND SULANKHE,D.K.(1981) CRC Crit Rev.Toxicol., 9, 21.

JERZYKOWSKI,T., MATUSZEWSKI, N., DIROZONSEK, N. AND WINTER, R. (1970) Neoplasm, 17, 255.

JONES, D.P., EKLOW, L., THOR, H. AND ORRENIUS, S.(1981) Arch. Biochem. Biophys., 210, 505.

KAPADIA,G.J.(1982) In: Oncology Overview on Naturally Occurring Dietary Carcinogens of Plant Origin, Ed.(International Cancer Research Data Bank Program, National Cancer Institute, Bethesda, Maryland).

KAPPUS,H. AND SIES,H.(1981) Experientia, 37, 1233.

KASAI.H., KUMENO,K., YAMAIZUMI,Z., NISHIMURA,S., NAGAO,M., FUJITA,Y., SUGIMURA,T., NUKAYA,H. AND KOSUGE,T.(1982) Gann, 73, 681.

KATSUI,N., YAGIHASHI,F., MURAI,A. AND MASAMUNE,T.(1982) Bull.Chem.Soc. Jpn., 55, 2424.

KINLEN,L.J.(1983) Br.Med.J., 286, 1081.

KINSELLA,A.R.(1982) Carcinogenesis, 3, 499.

KLAMERTH,O.L.(1968) Biochim.Biophys.Acta, 155, 271.

KORYCKA DAHL,M AND RICHARDSON,J.(1980) Biochim. Biophys.Acta. 610.229.

KRAMERS,P.G.N.(1975) Mutat.Res., 32, 81.

KRINSKY,N.I. AND DENEKE,S.M.(1982) J.Natl.Cancer Inst., 69, 205.

KURATOMI,K. AND KUBAYASHI,Y. (1977) Biochim. Biophys. Acta, 476, 207.

LEVIN,D.E., HOLLSTEIN,M., CHRISTMAN,M.F., SCHWIERS,E.A. AND AMES,B.N. (1982) Proc.Natl.Acad.Sci.USA, 79, 7445.

LIND,C., HOCHSTEIN,P. AND ERNSTER,L.(1982) Arch.Biochem.Biophys., 216, 178.

MAGA,J.A.(1979) CRC Crit.Rev.Food Sci.Nutr., 11, 355.

MAGEE, P.N. (1982) Banbury Report 12, Nitrosoamines and Human Cancer (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

MATHEWS-ROTH, M.M. (1982) Oncology, 39, 33.

MATSUMOTO,T., YOSHIDA,D., MIZUSAKI,S. AND OKAMOTO,H.(1977) Mutat.Res., 48, 279.

MAUGH, T.H. (1979) Science, 205, 1363.

McCORD, J.M. (1974) Science, 185, 529.

McCORD, J.M. AND DAY, E.D. (1978) FEBS Lett., 86, 13

MEYER, F.(1953) Bull.Soc.Chem.Biol., 35, 1395.

MILLER,E.C.(1978) Cancer Res., 38, 1479.

MONDER,C.(1967) J.Biol.Chem., 242, 4603.

MORI,H., SUGIE,S., YOSHIMI,N., ASADA,Y., FURUYA,T. AND WILLIAMS, G.M. (1985) Cancer Res., 45, 3125.

MORIMOTO,K., WOLFF,S. AND KOIZUMI,A.(1983) Mutat.Res.Lett., 119, 355.

NAGAO,M., HONDA,M.,SEINO,Y., YAHGI,T.,KAWACHI,T. AND SUGIMURA,T.(1977) Cancer Lett., 2, 235.

NAGAO,M., MORITA,N., YAHAGI,T., SHIMIZA,M., KUROYANAGI,M., FUKUOKA,M., YOSHIHARA, K., NATORI, S., FUGINO,T. AND SUGIMURA,T. (1981) Environ. Mutagen. 3, 401.

NAGAO,M., SUGIMURA,T. AND MATSUSHIMA,T.(1978) Ann.Rev.Genet., 12, 117.

NAGATA,C., KODAMA,M., KIMURA,T. AND AIDA,M. (1980) In: Carcinogenesis. Fundamental Mechanisms and Environmental Effects, Pullman,B., Ts'o, P.O.P and Gelbion, H.V., Eds. (D. Reidel Publishing Company, Boston) 43.

NAKASATO,F., NAKAYASU,M., FUJITA,Y., NAGAO,M., TERADA,M. AND SUGIMURA, T.(1984) Mutat.Res., 141, 109.

NAKAYAMA,T., KIMURA,T., KODAMA,M. AND NAGATA,C. (1983) Carcinogenesis, 4, 765.

NOSIKOV,V.V., BRAGER,E.A., KARLYSHEV,A.V., ZHUZE,A.L. AND POLYANOVSKY, D.L. (1976) Nucleic Acids Res., 3, 2293.

NOVI,A.M.(1981) Science, 212, 541.

O'CONNOR,P.J.(1981) J.Cancer Res.Clin.Oncol., 99, 167.

OSHSHIMA,H. AND BARTSCH,H. (1981) Cancer Res., 41, 3658.

PACKER,J.E., MAHMOOD,J.S.,MORA ARELLANO,V.O., SLATER,T.F., WILSON,R.L. AND WOLFENDEN,B.S. (1981) Biochem.Biophys.Res.Commun., 98, 901.

PAMUKCU,A.M., YALCINER,S., HATCHER, J.F. AND BRYAN, G.T.(1980) Cancer Res., 40, 3468.

PARIZA,M.W., LORETZ,L.J., STORKSON,J.M. AND HOLLAND,N.C.(1983) Cancer Res.(Suppl.), 43, 2444S.

PETO,R., DOLL,R., BUCKLEY,J.D. AND SPORN,M.B.(1981) Nature, 290, 201.

PETO,R. AND SCHNEIDERMAN (1981) In: Banbury Report 9.Qauntification of Occupational Cancer, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

PETRAKIS,N.L., GRUENKE,L.D. AND CRAIG,J.C.(1981) Cancer Res.,41, 2563.

PLAIN,H.L. (1955) Genetics, 40, 268.

PODHAJCER,O.L., FRIEDLANDER,M. AND CRAZIANI,Y.(1980) Cancer Res., 40, 1344.

PRYOR,W.A.(1976-1982) In: Free Radicals in Biology,Ed.(Academic Press, New York) 1.

PRYOR,W.A. (1988) Free Radical Biology and Medicine, 4, 219.

QUINLAN,G.J. AND GUTTERIDGE,J.M.C. (1987) Biochem.Pharm. 36, 3629.

RACKER,E. (1951) J.Biol.Chem., 190, 685.

RAHMAN,A., SHAHABUDDIN, HADI,S.M., PARISH,J.H. AND AINLEY,K. (1989a) Carcinogenesis (In press).

RAHMAN,A., SHAHABUDDIN, HADI,S.M. AND PARISH,J.H. (1989b) Biochem. J. (Communicated).

RAJRLAKSHMI,S., RAO,P.M. AND SARMA,D.S.R. (1978) Biochemistry, 17,4151

REDDY,J.K., WARREN,J.R., REDDY,M.K. AND LALWANI, N.D.(1982) Ann. N.Y. Acad. Sci., 386, 81.

RICHMOND,R., HALLIWELL, B., CHAUHAN, J. AND DARBRE, A. (1981) Anal. Biochem. , 118, 328.

RIDDLE,V. AND LORENZ,F.W.(1968) J.Biol.Chem., 243, 2718.

ROWLEY,D.A. AND HALLIWELL,B. (1982a) FEBS Lett., 138, 33.

ROWLEY,D.A. AND HALLIWELL,B. (1982b) FEBS Lett., 142, 39.

SALEM,H.M.(1975) Proc.Soc.Exp.Biol.Med., 96, 10.

SALIN,M.L AND McCORD,J.M. (1975) J. Clin. Invest. 56,1319.

SATO,J., WANG YEU-MING AND VAN EYS,J.(1980) J.Biol.Chem., 255, 20.

SCAIFE,T.F.(1969) Experientia, 25, 17.

SCHOENTAL,R.(1976) In: Chemical Carcinogens, Searle,C., Ed. (Am. Chem. Soc., Washington, D.C.) 629.

SCAIFE,T.F. (1969) Experientia, 25, 17.

SCHOENTAL,R. (1976) In: Chemical Carcinogen, Searle,C., Ed. (Am. Chem. Soc., Washingtin,D.C.) 629.

SCHOENTAL,R.(1982) Toxicol.Lett., 10, 323.

SIMIC,M.G. AND KAREL,M. (1980) In: Autoxidation in Food and Biological Systems, Eds.(Plenum, New York).

SINGER,B.(1983) Molecular Biology of Mutagens and Carcinogens (Plenum, New York) 65.

SPEIT,G. VAGEL,W. AND WOLF,M. (1982) Environ.Mutagen., 4, 135.

STICH,H.F. AND ROSIN,M.P. (1983) Life Sci., 32, 2641.

STICH,H.F., ROSIN,M.P., WU,C.H. AND POWRIE,W.D.(1981a) Mutat.Res., 90, 201.

STICH,H.F., ROSIN,M.P., WU,C.H. AND POWRIE, W.D.(1981b) Cancer Lett., 13, 89.

STICH,H.F. AND SAN,R.H.C.(1981) In:Short term Test for Chemical Carcinogens, Eds. (Springer-Verlag, New York).

SUGIMURA,T. AND NAGAO,M. (1979) CRC Crit. Rev. Toxicol., 189.

SUGIMURA,T. AND SATO,S.(1983) Cancer Res.(Suppl.), 43, 241S.

SUGUIRA,Y.(1979) Biochem.Biophys.Res.Commun., 90, 375..

SUN,L. AND SINGER,B. (1975) Biochemistry, 14, 1795.

SZENT-GYORGYI,A.(1977) Proc.Natl.Acad.Sci.USA, 74, 2844.

SZENT-GYORGYI,A., EGYUD,L.D. AND McLAUGHLIN,J.A. (1967) Science, 155, 539.

TAKAHAMA.U.(1983) Plant Physiol. 71, 598.

TAKAHAMA,U.(1984) Plant Physiol, 74, 852.

TAKAHAMA,U. (1985) Photochem. Photobiol., 42, 89.

TAKAHAMA, U., YOUNGMAN, R.J. AND ELSTNER, E.F (1984) Photobiochem. Photobiophys. 7, 175.

TAPPEL,A.L.(1980) In:Free Radicals in Biology, Pryor,W.A. Ed.(Academic Press, New York) 4, 1.

TATEISHI,N., HIGASHI,T., NARUSE,A., HIKITA,K. AND SAKAMOTO,Y.(1981) J. Biochem., 90, 1603.

TAUBER,A.I.(1982) Trends Biochem.Sci., 7, 411.

TAZIMA,Y.(1982) In: Environmental Mutagenesis,Carcinogenesis and Plant Biology. Klekowski, E.J. Jr., Ed.(Praeger, New York) 1, 68.

TOTH,B., NOGEL,D. AND ROSS,A.(1982) Br.J.Cancer, 46, 417.

TOTTER,R.J.(1980) Proc.Natl.Acad.Sci.USA, 77, 1763.

TRICHOPOULOS,D., PAPAPOSTOLOU,M. AND POLYCHRONOPOULOU,A.(1981) Int.J. Cancer, 28, 691.

TUYNS,A.J., PEQUIGNOT,G., GIGNOUX,M. AND VULLA,A.(1982) Int.J. Cancer, 30, 9.

VANDEN BERGHE,D.A.,VLIETINCK,A.J. AND VAN HOOF,L.(1986) Bull.Inst. Pasteur, 84. 3881.

VAN EYS,J., JUDGE,M.A., JUDO,J., HILL,W., BOZIAN,R.C. AND ABRAHAMS,S. (1962) J.Nutr., 76, 375.

VAN HOOF,L.,VANDEN BERGHE,D.A.,HATFIELD,G.M. AND VLIETINCK,A.J.(1984) Planta Med. 50, 513.

VARSHAVSKY,A.(1981) Cell, 25, 561.

VIDELA,L.A.,FERNANDEZ,V.,MARINIS,A.DE AND VALENZUELA,A.(1982) Biochem. Biophys.Res.Commun., 104, 9.

WARHOLM,M., GUTHENBERG,C., MANNERVIK,B. AND VON BAHR,C.(1981) Biochem. Biophys.Res.Commun., 98, 512.

WATSON,W.A.F.(1982) Mutat.Res., 103, 145.

WELSCH,C.W. AND AYLSWORTH,C.F.(1983) J.Natl.Cancer Inst., 70, 215.

WILLIAMS,G.M., MORI,H., HIRONO,I. AND NAGAO,M.(1980) Mutat.Res., 79,1.

WINSTON,G.W. AND CEDERBAUM,A.T.(1982) Biochem.Pharmacol., 31, 2301.

WOLFF,P.S., GARNER,A. AND DEAN,T.R.(1986) Trends Biochem.Sci., 11, 27.

WINTERBOURN,C.C.(1979) Biochem.J. 182, 625.

XUE,S.P.(1980) Proceedings of the Symposium on Recent Advances in Fertility Regulation (Beijing), 122.

YA,A., POTAPENKO,M.V., MUSHNIN,A.A.,KRASNOVSKY,Jr. AND SUKHORUKOV,V.L. (1982) Z.Naturforsch, 37, 70.

YUAN,P.M. AND GRACY,R.W.(1977) Arch.Biochem.Biophys., 183, 1.